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Nucleolar Proteins are Essential for Ribosome Biogenesis and Nuclear Homeostasis in *Drosophila melanogaster*

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NUCLEOLAR PROTEINS ARE ESSENTIAL FOR RIBOSOME BIOGENESIS
AND NUCLEAR HOMEOSTASIS IN *DROSOPHILA MELANOGASTER*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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in

The Department of Biological Sciences

by

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Abstract

Four nucleostemin-like proteins (NS1-4) were identified previously in *Drosophila melanogaster*. NS1 and NS2 are nucleolar proteins, while NS3 and NS4 are cytoplasmic. Here we show that NS2 is homologous to the human nucleostemin-like protein, Ngp1, and that endogenous NS2 and exogenous GFP-NS2 enriches within peripheral regions of nucleoli in larval polyploid midgut cells. Like NS1, NS2 depletion in midgut cells blocked the release of 60S ribosomal subunit as detected by the accumulation GFP-RpL11 within nucleoli, and this likely led to a general loss of 60S subunit proteins as shown by immunoblot analyses. At ultrastructural level, nucleoli in midgut cells depleted of NS2 displayed enlarged nucleolar granular components (GCs) not only on the nucleolar periphery, but interspersed within dense fibrillar components (DFCs). Depletion of NS2 also caused nucleolar stress: larval midgut cells displayed prominent autophagy where mitochondria were found enclosed by isolation membranes derived from the rER. Larval imaginal wing disc cells depleted of NS2 responded by inducing apoptosis as marked by anti-caspase 3 labeling; loss of these cells resulted in defective adult wings. We conclude that nucleolar proteins NS1 and NS2 have similar but non-overlapping roles in the final maturation or nucleolar release of 60S ribosomal subunits.

Nopp140, the nucleolar and Cajal body (CB) phosphoprotein of 140 kDa, is considered involved in several steps of ribosome biogenesis. In *Drosophila*, loss of Nopp140 causes redistribution of fibrillarin, the C/D small nucleolar ribonucleoprotein (snoRNP) methyltransferase; correspondingly, the 2'-O-

methylation of ribosomal RNA (rRNA) was reduced. *Nopp140* knockout also leads to a loss of cytoplasmic ribosomes and a significant drop in protein translation. Here we show that coilin, an essential component of CB, redistributed in the *Nopp140*^{-/-} cells by immunofluorescence microscopy. Both immunofluorescence microscopy and Western blot analyses showed that the coilin protein level was elevated, and RT-PCR supported the results by showing coilin mRNA increased approximately two fold in *Nopp140*^{-/-} larvae. NS1 and NS2 were also found to redistribute from the nucleolus upon *Nopp140* gene knockout. In summary, the phenotypes described in Chapter 3 indicate that Nopp140 participates in several aspects of ribosome biogenesis, and it is essential to maintain nuclear homeostasis.

Chapter 1

Literature Review

Nucleolus

The nucleolus was first described properly but independently by Wagner in 1835 and Valentin in 1836 (Pederson, 2011). Almost a hundred years later, the nucleolus was identified as a specific chromosomal locus by Heitz in 1931 and by McClintock in 1934. The nucleolus was then discovered in the 1960's as the site of ribosome biogenesis (Pederson, 2011).

Viewed by electron microscopy, a typical metazoan nucleolus contains three distinct compartments—the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The primary function of nucleolus is ribosome assembly. The key steps of the process are transcription of pre-rRNA from rDNA; association of the snoRNAs with the pre-RNA for pseudouridylation and methylation of the pre-rRNA; cleavage of the pre-rRNAs into mature ribosomal RNAs—18S, 5.8S and 28S; association of ribosomal proteins and the 5S rRNA, assembly into pre-ribosomal particles; and export of the nearly mature ribosomal particles from the nucleolus to the cytoplasm (Olson and Dundr, 2005). Transcription of rRNAs occurs at the border between the FC and the DFC, early modification of the pre-rRNA occurs in the DFC, and late processing occurs in the GC.

Besides rRNA transcription, processing and ribosome assembly, the nucleolus also participates in many other aspects of the cell function (Pederson, 1998). A possible connection between the nucleolus and mRNA processing was discovered in the 1960s (Sidebottom and Harris, 1969). Later, spliced *c-myc*

mRNA (intron 1 absent) was discovered accumulating in the nucleolus in several different types of mammalian cells (Bond and Wold, 1993). In addition, the human retroviral proteins involved in mRNA transport—the Rex protein of HTLV-I and the Rev protein of HIV—localize predominantly in the nucleolus (Cullen et al., 1988; Siomi et al., 1988). Recently, the mRNA encoding insulin-like growth factor 2 (IGF2), which is a regulator of myoblast proliferation and differentiation, is abundant and spliced in the nucleolus (Reyes-Gutierrez et al., 2014). Besides processing of these particular mRNAs, the nucleolus is important in processing other types of RNAs. One study showed that the nucleolus was involved in the processing of the Signal Recognition Particle (SRP) RNA and the initial assembly of this ribonucleoprotein complex (Jacobson and Pederson, 1998). In addition, the nucleolus also plays a role in processing the U6 snRNA (Pederson, 1998), and the transcription and processing of tRNA (Phizicky and Hopper, 2010). The nucleolus also participates in cell cycle progression (Ma and Pederson, 2007), aging (Guarente, 1997; Comai, 1999), and stem cell and cancer cell maintenance (Tsai and McKay, 2002; Beekman et al., 2006).

Assembly and disassembly of the nucleolus are regulated in a time- and space-dependent manner during each mitosis (Hernandez-Verdun, 2011). Nucleolar assembly starts at telophase of mitosis (Hernandez-Verdun, 2011). Both the activation of rDNA transcription and the recruitment and activation of pre-rRNA processing complexes are required for nucleolar assembly (Hernandez-Verdun et al., 2002). Nucleoli disassemble at the beginning of mitosis. At the beginning and during mitosis, CDK1-cyclin B phosphorylates

several Pol I-specific transcription factors, such as SL1 (Heix et al., 1998) and TTF1 (Sirri et al., 1999); the phosphorylation of Pol I components suppresses rDNA transcription and then nucleolar processing components are phosphorylated and released from the nucleolus (Peter et al., 1990). At the end of prophase of mitosis, the nuclear envelope breaks down and the chromosomes are condensed (Guttinger et al., 2009). The nucleolus is disassembled, but all the nucleolar components are stored in different cellular locations during mitosis (Hernandez-Verdun, 2011).

Nucleoli form around the clusters of tandemly repeated rDNA genes—the nucleolar organizing regions (NORs) (Leung et al., 2004). In addition, formation of prenucleolar bodies (PNBs) and the localization of the RNPs complexes from the PNBs to the rDNA transcription sites are also required for the assembly of the nucleolus (Stevens, 1965; Ochs et al., 1985a; Hernandez-Verdun, 2011). At late mitosis, the nucleolar components are subsequently transferred into the reassembling nucleolus as it reforms around the NORs (Dundr et al., 2000; Savino et al., 2001).

Ribosome Biogenesis

Ribosomes are the complex cellular machines that synthesize proteins by decoding the information carried by messenger RNA. Intact 80S ribosomes in eukaryotic cells consist of two ribonucleoprotein subunits. The 40S small subunit (SSU) contains the 18S rRNA and approximate 30 different small subunit proteins (RPSs). The 60S large subunit (LSU) contains 28S, 5.8S and 5S rRNAs and around 50 large subunit proteins (RPLs). Ribosome biogenesis is a rapid

and complicated process which includes the transcription of rRNA in the nucleolus, pre-rRNA modification and processing, association between rRNAs and ribosome proteins, and export of pre-ribosomes from the nucleus to the cytoplasm (Woolford and Baserga, 2013).

Drosophila melanogaster contains about 150 tandemly repeated rRNA coding genes in each nucleolus organizer, one on the X chromosome and one on the Y chromosome (Long and Dawid, 1979). A typical rRNA transcription unit contains an external transcribed spacer (ETS), an 18S region, a 5.8S region, and an additional 2S region in *Drosophila* which are flanked by internal transcribed spacers I and II (ITS I and ITSII), and a 28S region (Long and Dawid, 1980). Around half of the 28S rRNA coding regions have retro-transposon insertions; these include two distinct lineages of site-specific non-long-terminal-repeat retrotransposons—R1 and R2 (Glover and Hogness, 1977; Wellauer and Dawid, 1977; Jakubczak et al., 1990). However, the transcription level of rDNA with R1 and R2 insertions is much lower than that of rDNA without insertions (Ye and Eickbush, 2006). The mature 18S rRNA assembles with RPSs into the small ribosomal subunit, while the mature 5.8S and 28S rRNAs assemble with RPLs and the 5S rRNA to form the large ribosomal subunit.

The mechanism of ribosome biogenesis is best studied in yeast, so the yeast model is used here to demonstrate ribosome assembly. To ensure accurate construction of ribosomes, more than 200 proteins, such as ribosome assembly factors, endonucleases and exonucleases, and more than 70 small nucleolar RNAs are involved. The assembly of ribosomes starts with transcription

of pre-rRNA. The *Saccharomyces cerevisiae* rDNA consists of approximately 150 tandem repeats on chromosome XII (Figure 1.1) (Thiry and Lafontaine, 2005). RNA polymerase I transcribes the 35S pre-rRNA which is processed into mature 18S, 5.8S and 25S (slightly different from 18S, 5.8S and 28S found in higher eukaryotic species) (Thiry and Lafontaine, 2005). The 35S pre-rRNA contains a 5' external transcribed spacer (5' ETS), two internal transcribed spacers 1 and 2 (ITS1 and ITS2), and a 3' external transcribed spacer (3' ETS) (Figure 1.1). The mature 18S rRNA will assemble into the SSU, while the mature 5.8S and 28S rRNAs will join the LSU. The third rRNA in the LSU is transcribed by RNA polymerase III in the opposite direction. The 5S genes are located between rDNA genes in yeast, but in metazoans the 5S genes are tandemly repeated and located at other genetic loci that remain outside the nucleolus.

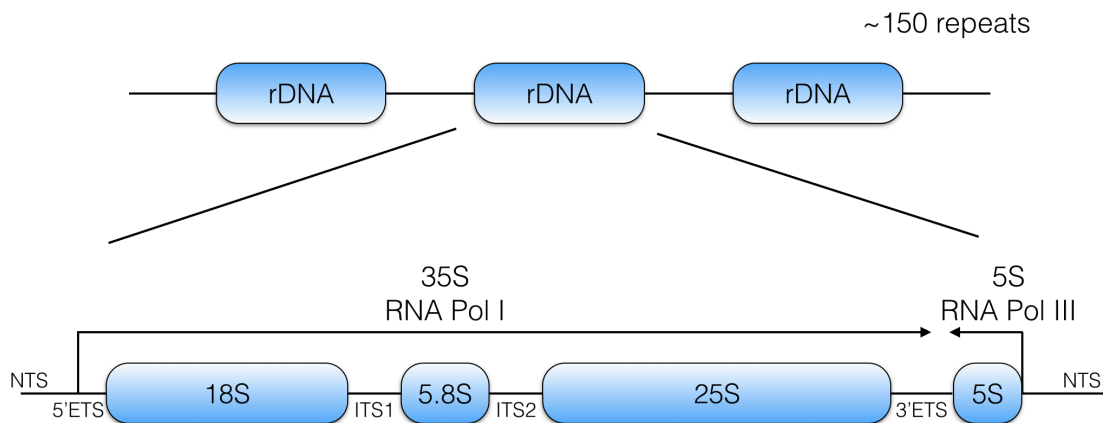


Figure 1.1 Structure of the rDNA in *S. cerevisiae*. The rDNA repeats (150–200) are located on chromosome 12. A single repeated unit is transcribed by RNA polymerase I (RNA pol I) (from left to right) to synthesize the 35S primary pre-rRNA transcript that will be processed to the mature 18S, 5.8S, and 25S rRNAs. RNA polymerase III (RNA pol III) synthesizes the 5S rRNA from right to left. NTS, nontranscribed spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer. (Adapted from Woolford & Baserga, 2013)

The four transcribed spacers in the pre-rRNA are cleaved by endonucleases and exonucleases (Venema and Tollervey, 1995). Cleavage of the 35S pre-rRNA usually occurs first either at sites A_0 or A_1 in the 5' ETS, or at site A_2 in the ITS1 (Figure 1.2). The 5' ETS is removed in two steps: 33S pre-rRNA is produced after the cleavage at site A_0 , and later 32S pre-rRNA is generated after the cleavage at site A_1 (Hughes and Ares, 1991; Beltrame et al., 1994). Cleavage at site A_2 in ITS1 generates the 20S pre-rRNA and 27SA₂ pre-rRNA (Udem and Warner, 1972). Alternatively, ITS1 can also be cleaved at the A_3 site, generating 23S and 27SA₃ pre-rRNAs (Lygerou et al., 1996). The 20S pre-rRNA and 27SA₂ pre-rRNA are packaged into a 43S pre-rRNP (pre-ribosomal ribonucleoprotein) and a 66S pre-rRNP respectively (Udem and Warner, 1973; Trapman et al., 1975). The 43S pre-rRNP is exported from the nucleolus to the cytoplasm, and during the transport, the mature 18S rRNA is produced by the cleavage of the remaining ITS1 sequence at site D (Fatica et al., 2003). The processing of the 66S pre-rRNP is more complicated than the 43S, and there are two alternative pathways to process the 27SA₂ pre-rRNA in the 66S pre-rRNP. Approximately 85% of 27SA₂ pre-rRNA is processed in the first pathway, in which part of ITS1 is removed at site A_3 (Shuai and Warner, 1991; Lindahl et al., 1992; Lygerou et al., 1996). The remaining ITS1 is cleaved later to generate 27SB_S pre-rRNA (Henry et al., 1994). Then the 27SB_S pre-rRNA is cut at the C_2 site to produce 7S_S and 25.5S pre-rRNAs, which are then processed into mature 5.8S_S and 25S pre-rRNAs in several steps. In contrast, the other ~15% of 27SB_S pre-rRNA is cut at site B1_L to form the 27SB_L pre-rRNA. As with the

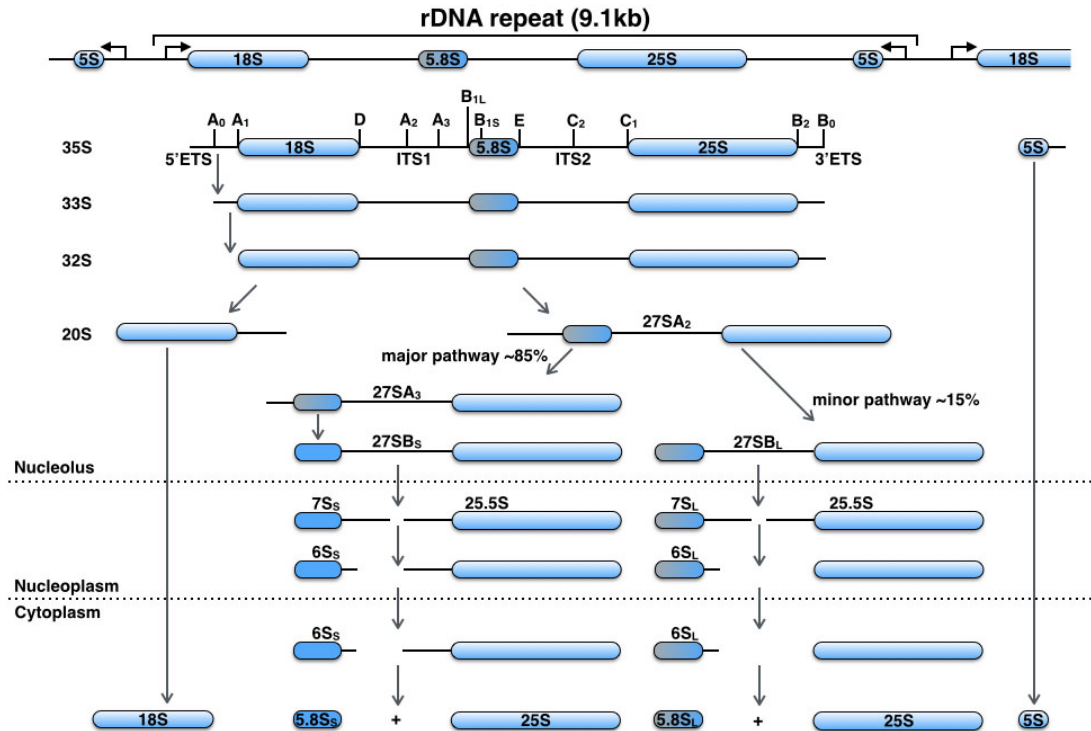


Figure 1.2 Pathway of yeast pre-rRNA processing. The 35S pre-rRNA is transcribed by RNA polymerase I and contains 18S, 5.8S, and 25S rRNAs flanked and separated by external and internal transcribed spacers (ETS and ITS, solid lines). Pre-5S rRNA is transcribed by RNA polymerase III. Spacer sequences are removed from pre-rRNA by a series of endonucleolytic and exonucleolytic processing steps, all within assembling ribosomes. Each processing site is indicated. Processing begins in the nucleolus of the cell, but later steps occur in the nucleoplasm and cytoplasm. (Adapted from Woolford & Baserga, 2013)

27SB_S pre-rRNA, the 27SB_L pre-rRNA is also cleaved at site C₂ producing 7S_L and 25.5S pre-rRNAs, both of which are processed into mature 5.8S_L and 25S pre-rRNAs in the end (Geerlings et al., 2000). The mature 5.8S_S and 5.8S_L rRNAs differ by 6 nt at their 5' ends (Henry et al., 1994; Mitchell et al., 1996). The processing of pre-rRNA can occur concurrently with transcription of pre-rRNA (Woolford and Baserga, 2013).

The pre-rRNAs are heavily 2'-O-ribose methylated and pseudouridylated by small nucleolar ribonucleoproteins (snoRNPs). Like cleavage of pre-rRNA, these modifications can also occur cotranscriptionally on the pre-rRNAs (Osheim et al., 2004; Kos and Tollervey, 2010). These modifications in the sequences of mature rRNAs are important for optimal function of ribosomes (Decatur and Fournier, 2002), and ribosomes that lack these modifications have lower translation efficiency (Baxter-Roshek et al., 2007; Liang et al., 2007; Jack et al., 2011). In addition, misdirection of these modifications can impair ribosome activities (Liu et al., 2008). The snoRNPs consist of a snoRNA and several proteins.

The snoRNPs are classified by their snoRNA sequence motifs (Kiss et al., 2010; Watkins and Bohnsack, 2012). The box C/D snoRNPs catalyze 2'-O-ribose methylation, while the box H/ACA snoRNPs catalyze pseudouridylation (Balakin et al., 1996; Watkins and Bohnsack, 2012). The box C/D snoRNAs consist of conserved box C, C', D, and D' motifs, and guide sequences of 10-21 nt that base pair with target rRNA sequences (Woolford and Baserga, 2013). There are four protein components in the box C/D snoRNPs—fibrillarin/Nop1, Nop56, Nop58, and snoSnu13; fibrillarin/Nop1 is the methyltransferase that catalyzes 2'-O-methylation (Singh et al., 2008). The methylation is directed by the guide RNA sequence; methylation occurs on the ribose of the nucleotide base paired with the 5th nt upstream of the box D or D' sequence (box D+5 rule) (Woolford and Baserga, 2013). The box H/ACA snoRNAs contain a conserved H (hinge) box followed by the conserved ACA box and 4-8 nt guide sequence that base pairs

with the target rRNA (Kiss et al., 2010; Watkins and Bohnsack, 2012). There are four protein components in the box H/ACA snoRNPs—Cbf5, Gar1, Nop10 and Nhp2. Among these proteins, Cbf5 is the pseudouridine synthase (Woolford and Baserga, 2013). Orthologs of Cbf5 include dyskerin in humans and Nop60 in *Drosophila*.

Because of the importance in assembly and function of ribosomes, most ribosomal proteins (r-proteins) are essential for yeast growth (Steffen et al., 2012). Even non-essential r-proteins play important roles in ribosome maturation and function (Baronas-Lowell and Warner, 1990; Briones et al., 1998; DeLabre et al., 2002; Babiano et al., 2012; Steffen et al., 2012). All r-proteins directly interact with rRNA in mature ribosomes, and most of these r-proteins are exposed on the surface of the ribosomes, serving as direct binding sites for assembly and interaction with translation factors (Woolford and Baserga, 2013).

All the processing and modifications of the different pre-ribosomal intermediates occur during their assembly within the nucleolus and their export from the nucleolus to the cytoplasm. Several proteins including the nuclear export factor Crm1, the Ran GTPase, Gsp1, and some nucleoporins are found in the export machinery; these proteins are necessary for export of both pre-40S and pre-60S subunits (Hurt et al., 1999; Moy and Silver, 1999; Stage-Zimmermann et al., 2000). Two proteins, Ltv1 and Pno1/Dim2, serve as adaptors for nuclear export of pre-40S ribosomes, and both of them contain of Nuclear Export Sequences (NES) sequences that can bind Crm1 (Seiser et al., 2006; Vanrobays et al., 2008). The export of 60S pre-ribosomes requires at least five

proteins; they are Arx1, Bud20, Ecm1, Mex67, and Nmd3 (Ho et al., 2000; Gadai et al., 2001; Bradatsch et al., 2007; Yao et al., 2007; Bassler et al., 2012). Among these proteins, Nmd3 serves as the export adaptor, interacting with both the pre-60S subunit and the export factor, Crm1 (Ho et al., 2000; Gadai et al., 2001).

GTPases involved in ribosome biogenesis

Many GTPases are required for ribosome biogenesis. GTPases are enzymes that bind and hydrolyze GTP to regulate crucial cellular processes. Most GTPases contain seven β -strands, known as GTP binding motifs G1-G7, which properly position the triphosphate moiety of a bound GTP. The GTPases can be divided into two classes based on their sequence and structure. One class contains the TRAFAC (for translation factor-related) GTPases, which includes the GTPases involved in translation, signal transduction and intracellular transport; the other class is SIMIBI, which has three large subclasses, the signal recognition GTPases, the MinD superfamily and the BioD superfamily.

Nucleostemin, the main focus of this dissertation, belongs to YlqF/YawG GTPase family, which is in the TRAFAC class. GTPases in YlqF/YawG family lack β strands 6 and 7 and have an additional N-terminal β strand compared to other GTPases. In addition, YlqF/YawG family GTPases have circularly permuted GTP binding motifs (Leipe et al., 2002). These GTP binding motifs (MMR1_HSR1 GTP-binding domain), contain five GTP-binding motifs in a circularly permuted order—G5* (conserved G5 variant motif), G4, G1, G2, and G3 (Tsai and Meng, 2009) (Figure 1.3)

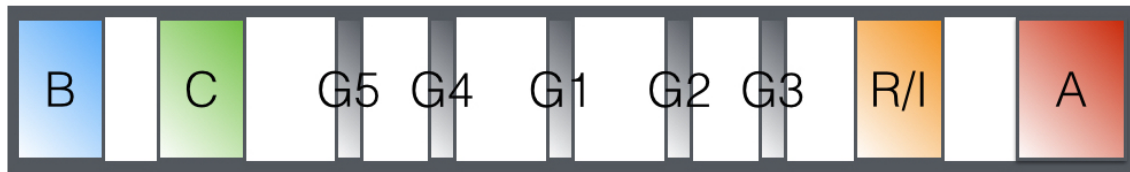


Figure 1.3 Protein structure of mammalian nucleostemin. Mammalian nucleostemin has a N-terminal basic domain (B), which is followed by a coiled-coil domain (C). After the coiled coil domain are the five GTPase binding motifs in a circularly permuted order (G5, G4, G1, G2, and G3). Following the last GTP binding motif is a RNA-binding domain (R) which is also an inhibitory domain (I) that blocks nucleolar retention of NS when GTP is not bound. At the carboxy terminus, mammalian nucleostemin contains an acidic region (A). (Adapted from Tsai and Meng, 2009)

There are at least four GTPases involved in yeast ribosome biogenesis.

Nog1 is a nucleolar GTP-binding protein found in eukaryotes ranging from trypanosomes to humans. Nog1 is associated with the pre-60S ribosome, and disruption of Nog1 causes nucleolar accumulation of 60S subunits and dramatic decrease in the levels of free 60S particles (Jensen et al., 2003). Studies also show that Nog1 is important for the processing of pre-rRNA: a Nog1 mutant causes accumulation of pre-rRNA intermediates and disrupts the maturation of ribosomes (Jensen et al., 2003; Saveanu et al., 2003; Fuentes et al., 2007).

A cytoplasmic protein Lsg1, like Nog1, is found associated with free 60S ribosomal subunits and the nuclear export adapter Nmd3. Both proteins are important for the 60S subunit export from the nucleolus. The role of Lsg1 in nuclear export is indirect since the protein is cytoplasmic. Lsg1 likely helps to recycle the export factors back to the nucleolus (Kallstrom et al., 2003).

Nog2 is another YlqF/YawG family GTPase. In contrast to loss of Nog1, which causes nucleolar accumulation of 60S subunits, loss of Nog2 causes pre-60S ribosomal complexes to accumulate in the nucleoplasm (Saveanu et al., 2001). Nog2 also plays an essential role in pre-rRNA processing. Depletion of Nog2 causes a concomitant increase in the levels of both 27SB_S and 7S_S precursors and disturbs 5.8S and 25S rRNA maturation (Saveanu et al., 2001). In addition, another YlqF/YawG family GTPase, Nug1, is involved in export of 60S subunits (Bassler et al., 2006).

Mammalian Nucleostemin

The *Nucleostemin* gene (*NS*) was first identified among several genes whose expressions were increased in MCF-7 human breast cancer cells by 17 β -estradiol treatment (Charpentier et al., 2000). The *NS* gene was then discovered as one of several genes that preferentially expressed in undifferentiated neural stem cells compared to their differentiated progeny (Tsai and McKay, 2002). There are three members in the mammalian nucleostemin family—nucleostemin, GNL3L and Ngp-1. They all belong to YlqF/YawG GTPase subfamily, in which all proteins contain a MMR1_HSR1 motif, which contains the circular permuted GTPase motifs (Leipe et al., 2002). The MMR1_HSR1 proteins in eukaryotes can be found throughout the nucleolus, nucleus and cytoplasm (Reynaud et al., 2005). Compared to other proteins in the YlqF/YawG GTPase subfamily, all three members of the mammalian nucleostemin family are closely related to each other and show a predominantly nucleolar localization (Tsai, 2011). A genome-wide search reveals the existence and transition of the nucleostemin subfamily genes

in phylogeny: almost all vertebrate species have nucleostemin, GNL3L, and Ngp-1 as separate genes (Tsai and Meng, 2009). However, for both nucleostemin and GNL3L, invertebrate genomes have only one ortholog. These are organisms with complete genome information, including *D. melanogaster* (NS1), *C. elegans* (K01C8.9), *S. cerevisiae* (Nug1) and *S. pombe* (Grn1). All these non-mammalian proteins have similar homology to human nucleostemin and GNL3L (Tsai and Meng, 2009). In contrast, Ngp-1 is highly conserved, from yeast to human. These studies suggest that mammalian nucleostemin and GNL3L may share a common invertebrate ancestor (Tsai and Meng, 2009).

Nucleostemin protein structure

The nucleostemin family proteins differ from other GTPase family proteins in their MMR1_HSR1 GTP-binding domain, which contains five GTP-binding motifs in a circularly permuted order—G5* (conserved G5 variant motif), G4, G1, G2, and G3 (Tsai and Meng, 2009) (Figure 1.3). Besides the GTP-binding domain, most proteins in the nucleostemin family consist of a basic domain and a coiled-coil domain at the N-terminus, an RNA-binding motif (inhibitory domain), followed by an acidic domain at the C-terminus (Figure 1.3). The N-terminal basic domain is required for the short term nucleolar targeting of human NS (Tsai and McKay, 2002, 2005). The basic domain also mediates the interaction between human NS and p53. The GTP-binding domain is required for appropriate nucleolar structures. Mutants lacking the GTP-binding domain disrupt cell cycle regulation and induce cell death. The inhibitory domain and acidic domain are required for long-term nucleolar retention of NS. Deletion of the coiled-coil

domain does not affect nucleolar distribution of NS nor the structure of nucleoli (Tsai and McKay, 2002, 2005).

Expression of Nucleostemin

Mammalian nucleostemin is found in different types of stem cells, including neural stem cells, embryonic stem cells, mesenchymal stem cells, primordial germ cells, and putative cardiac stem cells (Tsai and McKay, 2002; Baddoo et al., 2003; Liu et al., 2004; Beekman et al., 2006; Kafienah et al., 2006; Ohmura et al., 2008; Siddiqi et al., 2008). Nucleostemin is also found highly expressed in some human tumor and cancer cell lines, such as tumor-initiating cells (TIC) and cancer stem cells (CSC) of mammary and brain tumors (Tsai and McKay, 2002; Tamase et al., 2009; Lin et al., 2010). In normal adult tissues, high expression of nucleostemin is found only in the testis (Tsai and McKay, 2002; Ohmura et al., 2008). However, the expression of nucleostemin can be reactivated in some regenerating adult lens and limb tissues of *Cynops pyrrhogaster* (Japanese fire belly newt) (Maki et al., 2007). The expression of nucleostemin drops significantly just before the terminal differentiation of stem cells or before dividing cells exit the cell cycle (Tsai and McKay, 2002; Yaghoobi et al., 2005). Thus, different levels of nucleostemin may play an important role in cell cycle control— making self-renewing or long-term dividing cells stay in the cell cycle, or perhaps leading differentiated cells to reenter the cell cycle (Tsai, 2011).

Localization of Nucleostemin

Like most nucleolar proteins, nucleostemin shuttles between the nucleolus and the nucleoplasm. Surprisingly, the nucleolar localization signal (NoLS) of nucleostemin contains two components—the N-terminal basic domain and the GTP-binding domain (Tsai and McKay, 2005; Meng et al., 2006). Nucleostemin requires the basic domain for transient nucleolar retention, while GTP-binding is required for long-term retention in the nucleolus (Tsai and McKay, 2005). The inhibitory domain can block the nucleolar localization of non-GTP-bound nucleostemin (Tsai and McKay, 2005). In addition, the nucleolar retention of non-GTP-bound nucleostemin is restored after the inhibitory domain is deleted (Tsai and McKay, 2005). When bound to GTP, nucleostemin with its intact basic domain has long-term interaction with other nucleolar components. This retards nucleostemin diffusing between the nucleolus and the nucleoplasm, thus causing prolonged nucleolar retention of nucleostemin (Figure 1.4). Conversely, in the absence of GTP-binding, the inhibitory domain can prevent the interaction between the basic domain and the other nucleolar components; this leads to fast diffusion of nucleostemin through the nucleolus (Misteli, 2005). Like nucleostemin, the other two members of nucleostemin family, GNL3L and Ngp-1, both contain an inhibitory domain that prevents their nucleolar accumulation (Meng et al., 2007). Similar to nucleostemin, the nucleolar retention of both proteins is regulated by their GTP-binding domains. The nucleolar retention of nucleostemin and Ngp-1 is long and requires the basic domain, whereas the

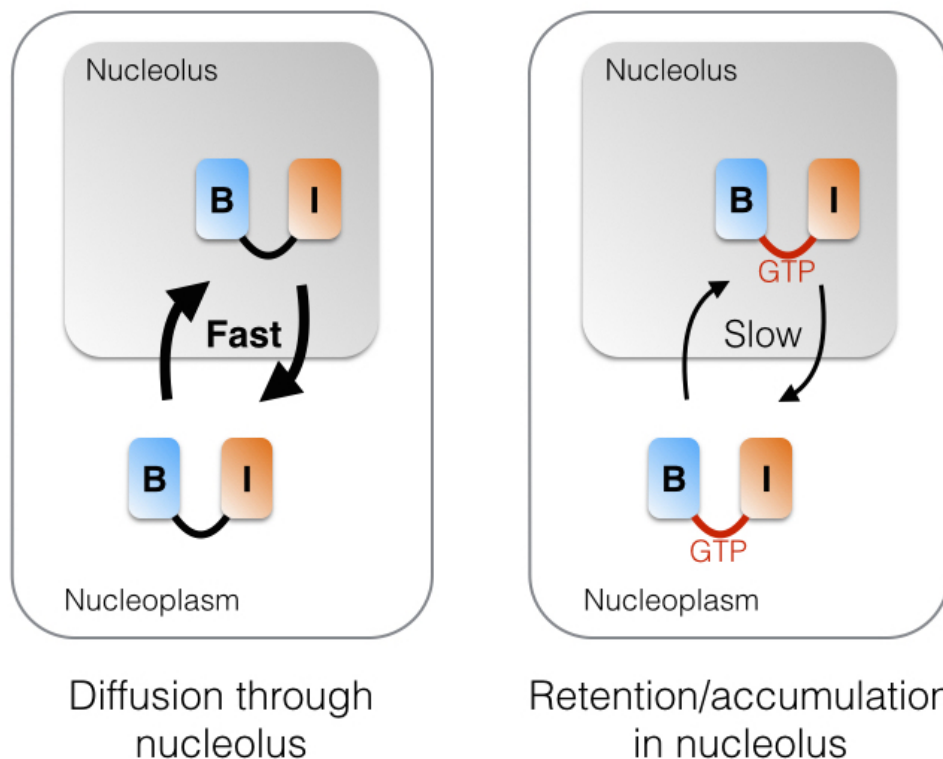


Figure 1.4 Nucleolar localization of nucleostemin depends on GTP-binding. (Left) In the absence of GTP binding, nucleostemin can diffuse through the nucleolus rapidly. (Right) Upon binding of GTP, the inhibitory action of the inhibitory domain (I) on basic domain (B) is relieved. When nucleostemin diffuses into the nucleolus, the basic domain is able to interact with other nucleolar components, which causes prolonged retention and accumulation of nucleostemin in the nucleolus. Hydrolysis of GTP reverses the stable binding, and nucleostemin dissociates from the nucleolus. (Adapted from Misteli, 2005)

nucleolar retention of GNL3L is short and requires both the basic domain and the coiled-coil domain (Meng et al., 2007).

Nucleostemin Interactions

The function of nucleostemin was determined through its interactions with other proteins. Nucleostemin can bind several proteins, such as p53, MDM2, RSL1D1, B23 and TRF1 (Tsai and McKay, 2002; Meng et al., 2006; Zhu et al., 2006; Ma and Pederson, 2007; Dai et al., 2008). p53 was the first protein found

to bind nucleostemin (Tsai and McKay, 2002). In mammals, p53 is a transcription factor and a key cell cycle regulator and tumor suppressor. In normal conditions, the p53 level is kept at low concentrations by MDM2 (murine and/or human double minute 2), which is an E3 ubiquitin ligase and targets p53 for degradation (Brooks and Gu, 2006). During nucleolar stress, proteins such as ribosomal proteins (RpL5, RpL11), the 5S rRNA (Donati et al., 2013; Sloan et al., 2013), and ARF (alternative reading frame) (Llanos et al., 2001) can interact with MDM2. As these components bind MDM2, they interrupt the interaction between p53 and MDM2. p53 is then stabilized and activates the cell cycle inhibitor gene *p21* and other p53-responsive genes, which lead to cell cycle arrest or apoptosis (James et al., 2014).

The cell death phenotype related to the over-expression of the GTP-binding domain of nucleostemin is p53-dependent (Tsai and McKay, 2002). However, the cell cycle arrest phenotype of nucleostemin depletion is either p53-dependent (Ma and Pederson, 2007) or p53-independent (Beekman et al., 2006; Nikpour et al., 2009). The interaction between p53 and nucleostemin is actually mediated by the p53 regulator, MDM2 (Dai et al., 2008; Meng et al., 2008). Over-expressed nucleostemin can bind to MDM2 directly and inhibit MDM2-mediated p53 ubiquitylation and degradation (Dai et al., 2008). In contrast, knockdown of nucleostemin by siRNA can cause nucleolar stress and release of ribosomal proteins L5 and L11 that then bind to MDM2 and inhibit MDM2 function (Dai et al., 2008). In both cases, p53 is activated and initiates G1 cell cycle arrest (Figure 1.5).

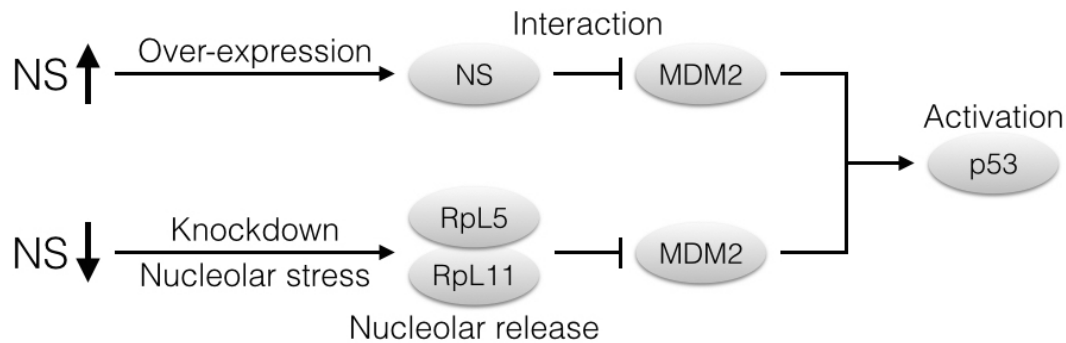


Figure 1.5 A nucleostemin (NS) model for cell cycle regulation. Nucleostemin is a highly dynamic nucleolar protein. Cell culture studies show that both over-expression and knockdown of NS cause p53 activation and subsequent cell cycle arrest and apoptosis. In this model, high levels of over-expressed NS cause NS to bind MDM2 and inhibit its p53 destabilization activity. Conversely, low levels of NS cause nucleolar stress, which releases Rpl5 and Rpl11 from the nucleolus. The released Rpl5 and Rpl11 can then bind and inhibit MDM2, to thus stabilize p53. (Adapted from Dai et al., 2008)

Nucleostemin also interacts with ARF (alternative reading frame). The ARF protein (14 kDa in human, 19 kDa in mouse) functions as a tumor suppressor to induce cell cycle arrest and apoptosis (de Stanchina et al., 1998; Palmero et al., 1998; Radfar et al., 1998; Zindy et al., 1998; Chen et al., 2010). In normal cells, ARF normally forms a stable complex with nucleophosmin (NPM, B23) and localizes in the nucleolus (Lindstrom and Zhang, 2006; Sherr, 2006). However, nucleolar stress releases ARF to the nucleoplasm where it binds and blocks MDM2 to activate p53 as do other nucleolar proteins, such as Rpl5, Rpl11 and nucleostemin (Weber et al., 1999; Llanos et al., 2001). ARF binds the central acidic region of MDM2 to inhibit its E3 ubiquitin ligase activity (Meulmeester et al., 2003). Nucleostemin regulates G1/S transition in a p53-dependent manner, and under this circumstance, nucleostemin is down

regulated by ARF (Ma and Pederson, 2007). In contrast, in p53 null cells, nucleostemin controls cell cycle progression by regulating the stability of ARF (Lo et al., 2015). Over-expression of nucleostemin can stabilize ARF by suppressing E3 ubiquitin ligase for ARF, and knockdown of nucleostemin leads to the decrease of ARF. Nucleostemin also has a stabilization function of nucleophosmin for ARF (Lo et al., 2015). Thus, there may be an alternative pathway in which stabilization of ARF as regulated by nucleostemin controls cell cycle progression in a p53-independent manner (Lo et al., 2015). What is more, nucleostemin can also bind to nucleophosmin that is essential for ribosome biogenesis. This suggests that nucleostemin may also play a role in ribosome biosynthesis (Lindstrom and Zhang, 2008; Ma and Pederson, 2008).

RSL1D1 (ribosomal L1-domain-containing 1), also called CSIG (cellular senescence-inhibited gene protein), is another nucleostemin binding partner (Meng et al., 2006; Ma et al., 2008). RSL1D1 promotes cell cycle progression by negatively regulating the tumor suppressor, PTEN (Ma et al., 2008). RSL1D1 co-localizes with nucleostemin in the same nucleolar region, but it displays a longer nucleolar residence time than nucleostemin (Meng et al., 2007). Both over-expression of RSL1D1 and loss of RSL1D1 reduce nucleolar nucleostemin levels (Meng et al., 2006). RSL1D1 has a higher binding affinity for nucleostemin and Ngp-1 than it does for GNL3L, which coincides with nucleostemin and Ngp-1 having a longer nucleolar residence time than GNL3L (Meng et al., 2007).

Besides the proteins that interact with nucleostemin described above, several studies have shown that TRF1 (telomerase repeat factor 1) can bind

nucleostemin. TRF1 is a component of telomere-capping complex, also known as shelterin or telosome (de Lange, 2005; Songyang and Liu, 2006). This complex plays a crucial role in maintaining the integrity of chromosomal ends (Martinez et al., 2009). Nucleostemin can bind TRF1 and negatively regulate its stability (Zhu et al., 2006). In contrast to nucleostemin, GNL3L, another nucleostemin family protein, can stabilize TRF1 by preventing its ubiquitylation and degradation (Zhu et al., 2009). Thus, nucleostemin and GNL3L show opposite regulation of TRF1 dimerization and its telomere binding ability (Meng et al., 2011b). Furthermore, nucleostemin can prevent DIF (dysfunction-induced foci) formation and telomere aberration in both telomerase-active and telomerase-inactive cells by promoting the association between PML-IV (one isoform of promyelocytic leukemia bodies) and SUMOylated TRF1 (Hsu et al., 2012).

Functions of Nucleostemin

Nucleostemin is essential for embryonic survival (Beekman et al., 2006; Zhu et al., 2006). Nucleostemin-null mouse embryos die around embryonic day 4, and these homozygous mutant blastocysts failed to enter S phase even in the absence of p53 (Beekman et al., 2006). Similar results were seen in another study that showed reduced growth and G2/M arrest in heterozygous nucleostemin-null mouse embryonic fibroblast cells (Zhu et al., 2006). Many studies have shown that nucleostemin plays an essential role in maintaining the continuous proliferation of cancer cells and stem cells (Tsai and McKay, 2002; Baddoo et al., 2003; Kafienah et al., 2006; Ma and Pederson, 2007; Dai et al., 2008; Meng et al., 2008; Ohmura et al., 2008; Qu and Bishop, 2012). Depletion

of nucleostemin disturbs the cell cycle progression either at the G1/S or G2/M transition, based on the types of tumor cells. Depletion of nucleostemin leads to G1/S arrest phenotype in some types of cells, such as U2OS (Dai et al., 2008), bone marrow-derived mesenchymal stem cells (Jafarnejad et al., 2008), HeLa cells (Sijin et al., 2004), prostate cancer PC-3 cells (Liu et al., 2004), and SW1710 bladder carcinoma cells (Nikpour et al., 2009). On the other hand, loss of nucleostemin causes G2/M arrest and a decrease in G1 phase cell percentage in other types of cells such as MEF cells (Zhu et al., 2006), U2OS cells (Meng et al., 2008), 5637 bladder carcinoma cells (Nikpour et al., 2009), and HeLa cells (Romanova et al., 2009). These conflicting data especially with HeLa cells may suggest that the impact of NS on cell cycle checkpoint control is indirect, but the exact mechanism is still unknown (Tsai, 2011). In contrast, over-expression of nucleostemin at a low-to-moderate level can promote cell proliferation (Zhu et al., 2006), and high levels of over-expression disturb cell cycle progression (Dai et al., 2008). A recent study showed that during mitosis, NS along with two other proteins—hTERT (human telomerase reverse transcriptase) and BRG1 (Brahma-related gene 1), assemble a TBN (hTERT/BRG1/NS) complex, which maintains heterochromatic repetitive elements (Maida et al., 2014). Loss of NS in HeLa cells disrupted heterochromatin formation and caused a mitotic arrest phenotype. Another study showed that the mammalian *NS* gene was a direct transcriptional target of the c-Myc proto-oncoprotein, a transcription factor that can either activate or repress gene expression (Zwolinska et al., 2012). In addition, the authors also demonstrated that NS was a critical regulator of tumor development

of c-Myc induced tumorigenesis. Haploinsufficiency of NS (NS level decreased by half) was sufficient to limit c-Myc induced lymphoma development *in vivo* in a p53-independent manner (Zwolinska et al., 2012).

Mammalian nucleostemin has been shown to play an important role in ribosome biogenesis in HeLa cells (Romanova et al., 2009). Nucleostemin forms a large protein complex (>700kDa), which includes the pre-60S ribosomal subunit. The complex also contains pre-rRNA processing proteins, such as Pes1, DDX21, and EBP2, as well as several ribosomal proteins (RpS6, RpS8, RpS24, RpL13, and RpL14) (Romanova et al., 2009). Knockdown of nucleostemin redistributes DDX21 and EBP2 from the nucleolus to the nucleoplasm or cytoplasm without changing the amounts of these two proteins. Loss of NS in HeLa cells also delays the processing of 32S pre-rRNA into 28S-rRNA, accompanied by a significant decrease of protein synthesis and a decrease of rRNA levels. Conversely, over-expression of nucleostemin substantially promotes the processing of 32S pre-rRNA (Romanova et al., 2009).

However, a recent study has shown that a 2-day nucleostemin knockdown triggers only DNA damage and cell cycle arrest without causing a significant disturbance of ribosome synthesis in human breast cancer cells (Lin et al., 2014). These authors believe that compared to disturbance of ribosome biogenesis, the DNA damage phenotype is likely related to nucleostemin knockdown, because the cells in the Romanova study were analyzed on the 5th day after two rounds of knockdown. In other words, the ribosomal biogenesis disruption is the terminal result of nucleostemin gene knockout or knockdown, rather than the direct result.

Some other studies show that nucleostemin has an essential role in maintaining the genomic integrity of stem and progenitor cells (Lin et al., 2013; Meng et al., 2013). Loss of nucleostemin also causes DNA damage and growth arrest in a p53-independent manner in mouse embryonic fibroblast cells. Nucleostemin is directly recruited to DNA damage sites where it helps recruit the core DNA repair protein, RAD51, and to stalled replication-induced DNA damage foci (Meng et al., 2013).

In contrast, depletion of GNL3L, another nucleostemin family protein, disturbs the processing of pre-rRNA without causing DNA damage in human breast cancer cells (Lin et al., 2014). These authors proposed that the divergent functions of nucleostemin and its paralog GNL3L appeared during the evolution from the invertebrates to vertebrates. However, nucleostemin and GNL3L share a common invertebrate ancestor (GNL3, NS1 in *Drosophila melanogaster*, Nug1 in *Saccharomyces cerevisiae*, NST-1 in *Caenorhabditis elegans*, and Grn1 in *Schizosaccharomyces pombe*), which suggests that the common invertebrate ancestor may have functions of both vertebrate nucleostemin and GNL3L.

Other Members of Nucleostemin Family

GNL3L was discovered in a screen for nucleostemin related genes (Du et al., 2006; Yasumoto et al., 2007). Nucleostemin and GNL3L exist as separate genes only in vertebrates (Tsai and Meng, 2009). They share a common ortholog—NS1 in *Drosophila melanogaster*, NST-1 in *Caenorhabditis elegans*, Nug1 in *Saccharomyces cerevisiae*, and Grn1 in *Schizosaccharomyces pombe* (Lin et al., 2014). GNL3L contains the MMR1-HSR1 domain, and the regular

basic, coiled coil, RNA-binding, and acidic domains. However GNL3L contains an extra coiled-coil domain at the C-terminus. Also unlike nucleostemin, GNL3L is expressed at a lower level in undifferentiated neural stem cells, but at a higher level in their differentiated progeny. GNL3L is also maintained in a higher level throughout neural development (Yasumoto et al., 2007). Compared with nucleostemin, GNL3L has a much shorter nucleolar retention and a higher nucleoplasmic intensity (Meng et al., 2007). The expression of GNL3L is linked to the level of nucleostemin. A compensatory upregulation of both GNL3L mRNA and GNL3L protein occurs after depletion of nucleostemin (Tsai and McKay, 2002; Huang et al., 2009).

Like nucleostemin, GNL3L can bind MDM2 to regulate its level (Meng et al., 2011a). The GTP-binding domain of GNL3L is required for the interaction between GNL3L and MDM2, which occurs in the nucleoplasm. GNL3L has the same function as nucleostemin in stabilizing MDM2 and preventing its ubiquitylation. Over-expression of GNL3L in a nucleostemin mutant can rescue the ubiquitylation of MDM2 caused by nucleostemin depletion (Meng et al., 2011a). Depletion of GNL3L causes G2/M arrest and upregulates specific p53 downstream target genes, namely *Bax*, *14-3-3 σ* , and *p21*.

Compared to nucleostemin, which can negatively regulate TRF1 (Zhu et al., 2006), GNL3L can bind and stabilize TRF1. The interaction between GNL3L and TRF1 in the nucleoplasm causes an increased homodimerization and telomeric association of TRF1 (Zhu et al., 2006), which has a negative impact on the telomere elongation (van Steensel and de Lange, 1997). GNL3L can also

inhibit TRF1 ubiquitylation by binding to FBX4, an E3 ubiquitin ligase for TRF1, thus stabilizing TRF1 (Zhu et al., 2006). As a result, GNL3L and nucleostemin show an opposite regulation of TRF1 and telomere length. Furthermore, another study shows that nucleostemin and GNL3L exhibit distinct functions in genome protection and ribosome synthesis respectively (Lin et al., 2014). Depletion of nucleostemin in human breast carcinoma cells causes rapid and substantial DNA damage in S phase cells, whereas GNL3L depletion does not lead to significant DNA damage. In contrast, GNL3L depletion remarkably disturbs ribosome production, while depletion of nucleostemin does not significantly affect total ribosome production (Lin et al., 2014). These findings suggest the functions of nucleostemin and GNL3L diverged as the vertebrates evolved (Lin et al., 2014). However, the common ortholog of nucleostemin and GNL3L in invertebrates might have functions of both vertebrate proteins.

Ngp1 is the third member of mammalian nucleostemin family. The cDNA clone of Ngp1 was isolated by screening human ductal breast tumor expression libraries with autologous patient serum (Racevskis et al., 1996). Ngp1 localized to the nucleolus and nucleolar organizer regions in different types of cells, such as seminiferous tubule cells, skeletal muscle cells, normal colon and colon tumor cells. Little is known however about the function of mammalian Ngp1. The homolog of mammalian Ngp1, Nog2/Nug2 in budding yeast was shown to be involved in ribosomal biogenesis (Saveanu et al., 2001). Nug2-depleted yeast cells showed a defect in 5.8S and 25S maturation, and a concomitant increase in the levels of both 27SB_S and 7S_S precursors. Upon loss of Nug2, the pre-60S

ribosomal complexes can leave the nucleolus, but are retained in the nucleoplasm (Saveanu et al., 2001). Another study showed that yeast Nug1p and Nug2p are separately required for the nuclear export of the pre-60S subunit; mutation in either gene inhibits the export of 60S subunit (Bassler et al., 2001). A recent study has shown that Nug2 binds the inter-subunit face of maturing nucleoplasmic pre-60S particles, which clashes with the binding site of Nmd3, a key pre-60S export adaptor (Matsuo et al., 2014). Nug2 holds the position and controls the proper time when it is released from pre-60S particles. Upon release of Nug2 the nuclear export adaptor protein, Nmd3, is recruited to the position. The GTP-binding state of Nug2 is important for its binding to the pre-60S particle, and its GTP hydrolysis is crucial for its release from the position and the subsequent recruitment of Nmd3 (Matsuo et al., 2014).

Nucleostemin in *Drosophila*

There are four members of the *Drosophila* nucleostemin family—NS1, NS2, NS3, and NS4. All four members share a similar protein structure, with permuted GTP-binding motifs (Kaplan et al., 2008). However, NS1 and NS2 are more closely related to the mammalian nucleostemin family. Both NS1 and NS2 localize in the nucleolus, whereas NS3 displays a cytoplasmic distribution (Kaplan et al., 2008). NS1, NS2, and NS3 are all essential for *Drosophila* development, and depletion of any of these three proteins causes larval lethality (Kaplan et al., 2008; Rosby et al., 2009; Matsuo et al., 2010; Hartl et al., 2013), while NS4 seems dispensable for viability (Kaplan et al., 2008) and has not been studied in detail thus far.

NS3 is related to mammalian and yeast Lsg1, which is a cytoplasmic protein, but still involved in ribosome biogenesis (Kallstrom et al., 2003). Yeast Lsg1 is involved in recycling Nmd3. Among all four proteins in the *Drosophila* nucleostemin family, only NS3 can rescue the yeast Lsg1 lethality phenotype (Hartl et al., 2013). Like yeast Lsg1, NS3 is also involved in ribosome biogenesis. NS3 plays an important role in exporting 60S and 40S subunits from the nucleus to the cytoplasm (Hartl et al., 2013). Knockdown of NS3 significantly reduces total cellular amounts of RpS6 and RpL13, and this role of NS3 in ribosome biogenesis is essential in all tissues (Hartl et al., 2013). In addition, NS3 in *Drosophila* controls global organism growth by regulating insulin signaling (Kaplan et al., 2008). Loss of NS3 elevates insulin level in insulin-producing cells and reduces peripheral insulin pathway activation, and thus disrupting larval growth. Reintroduction of NS3 relieves the accumulation of insulin in insulin-producing cells and rescues the growth defects.

NS1 is the closest ortholog to mammalian nucleostemin (Rosby et al., 2009), and is the common invertebrate ancestor of vertebrate nucleostemin and GNL3L (Tsai, 2011). NS1 localizes in the nucleoli of most larval and adult cells (Kaplan et al., 2008; Rosby et al., 2009). NS1 plays an essential role in ribosome biogenesis, and loss of NS1 leads to larval or pupal lethality. Depletion of NS1 blocks the nucleolar release of large ribosomal subunits and causes a loss of cytoplasmic ribosomes (Rosby et al., 2009).

NS2 is the other nucleolar protein in the *Drosophila* nucleostemin family (Kaplan et al., 2008; Matsuo et al., 2011); it is closely related to the human

nucleostemin-like protein, Ngp1. NS2 is essential for early eye development and cell survival (Matsuo et al., 2010). NS2 is also involved in ribosome biogenesis, and its depletion causes a reduction of the ratio of cytoplasmic rRNA to nuclear rRNA without affecting rRNA maturation (Matsuo et al., 2011). Loss of NS2 causes larval lethality.

It is important to point out that *Drosophila* lacks MDM2, ARF and nucleophosmin discussed above, thus the role and regulation of p53 in *Drosophila* varies from mammalian p53 (Jin et al., 2000; Sogame et al., 2003; Mogila et al., 2006; Moon et al., 2008). As a result, proteins in the *Drosophila* nucleostemin family might show different roles if any in regulating cell cycle progression and apoptosis.

This dissertation focuses on NS2 in *Drosophila*. Chapter 2 describes NS2 distribution and its function by RNAi knockdown of NS2.

Nopp140

Nopp140 (Nucleolar phosphoprotein of 140 kDa) is another nucleolar protein that is involved in ribosomal biogenesis. Like NS2, loss of Nopp140 also causes nucleolar stress. Our previous work showed that loss of Nopp140 leads to redistribution of fibrillarin from the nucleolus. Here we are interested in determining whether loss of Nopp140 has impacts on other nucleolar proteins that are involved in ribosomal biogenesis.

Nopp140 is conserved among eukaryotes, from yeast to human. Nopp140 was first identified in rat as a binding protein of the nuclear localization signal (NLS) of the SV-40 large T antigen (Meier and Blobel, 1990). Nopp140 localizes

in the fibrillar regions of nucleoli. The structure of Nopp140 includes the conserved N-terminal and C-terminal domains, and a large central region with alternating basic and acid regions (He and DiMario, 2011b). Human Nopp140 (p130) was first discovered as a novel human nucleolar phosphoprotein (Pai et al., 1995). Human p130 shows 74% identity to rat Nopp140. p130 localizes in the prenucleolar bodies during telophase, and in the nucleoli during interphase (Pai et al., 1995). p130 is heavily phosphorylated by casein kinase II (CKII) during interphase with hyper-phosphorylation by CDK1-cyclin B (also called M-phase promoting factor, MPF) occurring during mitosis. An isoform of p130 was found and it contains an insertion of ten amino acids in the fourth basic region (Pai and Yeh, 1996). Both human p130 isoforms (p130 α and p130 β) are co-expressed in different types of cells. But the transcript of p130 β is much less abundant than is the transcript of p130 α . Both isoforms show the same nucleolar localization pattern.

The cDNA encoding *Drosophila* Nopp140 was first found in an effort to recover *Drosophila* cDNAs encoding either nucleolin or Nopp140 through screening a stage 10-egg chamber cDNA library (Waggener and DiMario, 2002). There are two *Drosophila* Nopp140 isoforms, Nopp140-RGG and Nopp140-True. Nopp140-RGG contains a distinctive Arg-Gly-Gly (RGG) rich carboxyl domain (Waggener and DiMario, 2002). Similar RGG domains are often found in RNA binding proteins such as fibrillarin and vertebrate nucleolin (Lischwe et al., 1985; Ochs et al., 1985b). Nopp140-True is homologous to mammalian Nopp140. The C-terminus of Nopp140-True has 64% and 65% sequence identity with the

carboxy termini of rat and human Nopp140 respectively (He and DiMario, 2011b). The two *Drosophila* isoforms share the same amino-terminus and large alternating acidic and basic central domain. However, they differ in their carboxy-terminal domains due to alternative splicing (Waggener and DiMario, 2002). Both proteins contain casein kinase II (CKII) and Cdk1 (MPF) phosphorylation sites. In addition, both proteins show the same nucleolar localization when expressed in *Drosophila* Schneider II culture cells or transgenic fly cells (McCain et al., 2006).

Nopp140 is not required for nucleolar formation, but its over-expression disrupts nucleolar integrity and function (He and DiMario, 2011b). Depletion of the mammalian Nopp140 ortholog in *S. cerevisiae*—Srp40 (Meier, 1996) or *Drosophila* Nopp140 (Cui and DiMario, 2007) fails to disturb nucleolar structure. However, over-expression of full length human Nopp140 highly disrupts nucleolar integrity. The over-expression of hNopp140 caused the formation of a transcriptionally inactive spherical body occupying the entire nucleolus, in which the RNA polymerase I, fibrillarin, and hNopp140 were trapped (Chen et al., 1999). Over-expression of both *Drosophila* isoforms causes nucleoli to swell (hypertrophy) (Cui and DiMario, 2007).

Nopp140 functions as a chaperone for small nucleolar ribonucleoproteins (snoRNPs) (Yang et al., 2000). Rat NAP57 (dyskerin in humans, Cbf5 in yeast) was co-immunoprecipitated with Nopp140. This was the first indication that Nopp140 interacts with snoRNPs (Meier and Blobel, 1994). NAP57 is a pseudouridylase, a component of box H/ACA RNPs, which consists of four proteins and one of several box H/ACA guide RNAs (Lafontaine et al., 1998;

Watkins et al., 1998). Intact snoRNP complexes containing H/ACA guide RNAs were also immunoprecipitated with Nopp140 (Yang et al., 2000). However, Nopp140 is not required for the in vitro pseudouridylation by box H/ACA snoRNP complexes (Wang et al., 2002). Nopp140 might act as a scaffold for multiple snoRNPs as they modify the pre-rRNA. In addition, Nopp140's association with snoRNPs is dependent on its extensive CKII phosphorylation (Wang et al., 2002).

Compared to association with box H/ACA, the association between Nopp140 and C/D box is weaker (Yang et al., 2000). NAP65 and fibrillarin, two components of box C/D box snoRNPs, were identified in Nopp140 immunoprecipitates, but under less stringent conditions. Nopp140 was shown to associate with the specific U3 C/D box snoRNP complex (Watkins et al., 2004). Nopp140, along with two other assembly factors, TIP48 and TIP49, were found associated with both precursor and mature U3-containing snoRNPs in nuclear extracts, suggesting that Nopp140 serves as a snoRNP biogenesis factor in the nucleoplasmic phase of U3 RNP assembly (Watkins et al., 2004). What is more, the yeast ortholog of mammalian Nopp140, Srp40, also plays a greater role in box H/ACA snoRNP biogenesis than it does in the assembly of box C/D snoRNPs (reviewed by He and DiMario, 2011b).

Nopp140 and Cajal Bodies

Newly synthesized Nopp140 in transfected culture cells localizes first to nucleoli and then to Cajal bodies (CBs), suggesting that Nopp140 shuttles snoRNPs from the nucleoli to CBs (Isaac et al., 1998). Compared to newly synthesized Nopp140, mature Nopp140 shuttles between the nucleolus, CBs,

and cytoplasm (Bellini and Gall, 1999). Regardless of the difference between newly synthesized Nopp140 and mature Nopp140, it is well accepted that Nopp140 is the chaperone for snoRNPs between CBs and the nucleoli (He and DiMario, 2011b). The CB is the nuclear compartment that plays a role in spliceosome assembly, preassembly of transcription complexes, and processing of snoRNAs (Nizami et al., 2010).

Coilin is the signature marker protein for CBs (Nizami et al., 2010). Human and mouse coilin proteins are similar in size (62.6 and 62.3 kDa respectively), and they are highly conserved in sequence (Andrade et al., 1991; Tucker et al., 2000). In *Drosophila*, coilin has a molecular weight of 70.6 kDa, while it migrates at about 90 kDa on western blots (Liu et al., 2009). Coilin binds to the survival motor neuron (SMN) protein and to various Sm (a large complex of proteins involved in the biogenesis of snRNPs, containing SMN, snRNP core components and other proteins) and Lsm (Sm-like) proteins, indicating that coilin may play a role in assembly or modification of snRNPs (Hebert and Matera, 2000; Hebert et al., 2001; Xu et al., 2005). Coilin is also required for the formation of CBs in different organisms. However, coilin is not essential for viability: knockout of the *coilin* gene in the mouse causes a semi-lethal phenotype (Walker et al., 2009). Instead of normal CBs, there are three types of residual bodies that contain a subset of CB components in cultured cells in which the *coilin* gene has been depleted (Tucker et al., 2001; Jady et al., 2003). In *Drosophila*, homozygous null mutants for coilin are fully viable, but these mutants lack CBs as assayed by

either immunostaining or in situ hybridization (Beumer et al., 2008; Liu et al., 2009).

Nopp140 was first found in CBs by immuno-fluorescence microscopy (Meier and Blobel, 1994). Co-localization of Nopp140 and p80 coilin was confirmed later by immuno-electron microscopy (Vandelaer and Thiry, 1998). Yeast two hybrid showed that Nopp140 can interact with the amino terminus of p80 coilin (Isaac et al., 1998). Beside the interaction between Nopp140 and p80 coilin, little is known about the relationship between these two proteins.

This dissertation focuses on several nucleolar and nuclear proteins, NS2, Nopp140, coilin, and NS1 in *Drosophila melanogaster*. Chapter 2 describes the nucleolar function of NS2 and cell stress phenotypes that arise upon NS2 depletion. Chapter 3 further describes the function of Nopp140 by showing redistribution of NS1, NS2, and coilin caused by Nopp140 knockdown. This dissertation will describe how coilin is regulated by knockout of Nopp140 in *Drosophila*, and what other phenotypes are caused by Nopp140 depletion. The resulting hypothesis generated from this work is loss of nucleolar proteins not only induces nucleolar stress, but disrupts nuclear homeostasis.

Chapter 2

Loss of *Drosophila* Nucleostemin 2 Blocks 60S Subunit Release from Nucleolar Granular Components Leading to Stress Response

Introduction

The nucleostemin (NS) family of proteins belong to a larger group of YlqF/YawG GTPases that in prokaryotes and eukaryotes share a distinct peptide domain referred to as MMR1_HSR1 in which GTP binding motifs display a circularly permuted arrangement of G5', G4, G1, G2, and G3 as compared to conventional GTPases such as Ras (Daigle et al., 2002; Leipe et al., 2002; Reynaud et al., 2005). The more immediate NS family in humans consists of nucleostemin itself (HNS), GNL3L, and Ngp1, with LSG1 and MTG1 being more distant relatives (Tsai, 2011). While *Drosophila melanogaster* fails to express a true homologue of human NS, it does express homologues of GNL3, Ngp1, and LSG1, respectively referred to as NS1, NS2, and NS3 (Kaplan et al., 2008; Tsai, 2011, 2014).

Much of the work on *Drosophila* NS-like proteins has been on NS1 and NS3. We showed earlier that NS1 in *Drosophila* has intrinsic GTPase activity, enriches within the granular components of nucleoli, and is required for the final maturation and/or release of the large (60S) ribosomal subunit from nucleoli (Rosby et al., 2009). NS3 is the functional orthologue to yeast LSG1, a cytoplasmic factor that functions in the release of the nuclear export adaptor protein, Nmd3p, from the large ribosomal subunit once the subunit arrives in the cytoplasm (Kallstrom et al., 2003; Hedges et al., 2005). Initial studies on NS3 in *Drosophila* showed that it is required in at least a relatively small set of

serotonergic brain neurons that signal neighboring neurons to release insulin-like growth factors; loss of NS3 greatly impaired normal development (Kaplan et al., 2008). Hartl et al. (2013) then confirmed the GTPase activity for NS3, and demonstrated the more systemic role of NS3 in ribosome biogenesis. Very little is known about NS4, the fourth NS-like member in *Drosophila*.

This report focuses on NS2. Matsuo et al. (2010) initially used a combination of ethyl methanesulfonate-induced point mutations and RNA interference (RNAi) expressions to show that NS2 is required for normal cell survival and early eye development. Matsuo et al. (2011) then showed that NS2 has intrinsic GTPase activity with a K_m of $0.34 \text{ mM} \pm 0.04 \text{ mM}$ for GTP binding and a k_{cat} of $42.6 \text{ h}^{-1} \pm 2.6 \text{ h}^{-1}$ for GTP hydrolysis. In this same report, the authors described an accumulation of rRNA in the nuclei of larvae that were homozygous for *ns2*⁵¹, a null (Q14/Stop) mutation. Here we generated a new antiserum against NS2 and transgenic larvae that expressed GFP-NS2 or RNAi to further describe NS2 in terms of its nuclear location, and the cell stress phenotypes that arise upon its depletion. Loss of NS2 by RNAi inhibits the release of the large (60S) ribosomal subunit from the nucleolus, and we show a corresponding expansion of the nucleolar granular component (GC). We also describe autophagy in larval polyploid midgut cells and apoptosis in larval diploid imaginal wing disc cells that result from the depletion of NS2.

Materials and Methods

Fly stocks

Fly stocks were obtained from the Bloomington Stock Center at Indiana University unless otherwise noted. Lines used in this study included w^{1118} (Bloomington stock #3605) which we used as a wild-type control, the second chromosome balancer stock w^*/w^* ; *Sp1/CyO* originally from W. M. Saxton (Indiana University), the third chromosome balancer stock w^-/w^- ; *Scm^{Et50}e/TM3 Sb¹*, *Ser* originally from J. A. Simon (University of Minnesota) and abbreviated here simply as *TM3/Et⁵⁰*, the third chromosome balancer stock w^-/w^- ; *Sb/TM3, Ser, Act-GFP* (Bloomington stock #4534) abbreviated here as *TM3-GFP*, the homozygous *daughterless-GAL4* driver line (Bloomington stock #55849), and the larval wing disc-specific $P[w[+m^*] = GAL4]A9$ on the X chromosome (Bloomington stock #8761) abbreviated here as *A9-GAL4*. The *A9-GAL4* driver expresses GAL4 strongly in larval wing and haltere discs (Haerry et al., 1998). The $P\{UAS-mCherry-ATG8a\}$ line, D168 (second chromosome), was a kind gift from Thomas Neufeld (Chang and Neufeld, 2009). Transgenic flies that express GFP-RpL11 (our A3 line on the 3rd chromosome), and mRFP-RpS6 (our F1 line on the 3rd chromosome) were previously described (Rosby et al., 2009). All fly lines were maintained on standard fly food at room temperature (22-24° C).

Protein sequence alignments

The *Drosophila* NS2 protein sequence (AAF57834) was aligned with *Drosophila* NS1 (AAF55384), human NS (AAH01024) and human Ngp1

(AAH09250) using the CLC sequence viewer software, version 7.5. NCBI-BLAST and Flybase.org BLAST tools were also used to analyze the sequences.

Plasmid constructions and fly transformations

The full length *Drosophila* NS2 cDNA (SD10213; *Drosophila* Genomics Resource Center, Bloomington, IN) was amplified using 5'-CACCATGCCAAAGGTACGCAGCAC-3' as the forward primer and 5'-GCCTAGAGTGTAACGTAATATTTTACGCGTCCTTC-3' as the reverse primer. Purified PCR products were ligated into the *pENTR/D* TOPO vector for the Gateway System (Invitrogen, Carlsbad, CA) using a TOPO Cloning Kit. The NS2 cDNA inserted into *pENTR* was then recombined into the *pTGW* destination vector (N-terminal eGFP-NS2) (*Drosophila* Genomics Resource Center) for fly transformations.

GAL4-induced expression of RNAi in transgenic flies (Brand and Perrimon, 1993; Duffy, 2002) employed *pUAST* that contains GAL4 upstream activation sequences (*UASs*) and the *Hsp70* promoter. To express small interfering RNAs specific for NS2 transcripts, a 450-base pair NS2 cDNA fragment encoding the first 150 amino acids of NS2 was amplified using the forward primer (5'-GTATGGTACCTAGTAGATCTATGCCAAAGGTAC-3') and the reverse primer (5'-ATAATCTAGAAAGTCGAATTCCCCAAAGGTACTGTC-3'). The resulting PCR product contained flanking KpnI and BglII sites (underlined) at the 5' end, and XbaI and EcoRI sites (underlined) at the 3' end. The reverse fragment was first ligated into *pUAST* using the EcoRI and BglII sites, and then the forward fragment was ligated into the resulting vector using the KpnI and

XbaI sites. The first cDNA segment was ligated into the plasmid in reverse orientation to prevent any possible translation of a truncated NS2 product that could potentially produce dominant negative effects.

Plasmids *pTGW-NS2* (expresses GFP-NS2) and *pUAST-RNAi-NS2* (expresses RNAi specific for *NS2* transcripts) were sent to GenetiVision (Houston, TX, USA) for embryo injections to produce transgenic flies. Ten independent insertion lines that expressed GFP-NS2 were established. Insertions were mapped to the X, second, or third chromosomes by using standard segregation analyses. The *UAS-GFP-NS2.2* line was used in this study; it maps to the third chromosome. Flies homozygous for *UAS-GFP-NS2.2* were crossed to flies homozygous for the *da-GAL4* transgene (Bloomington stock #55849) to induce ectopic expression of GFP-NS2 in heterozygous progeny larvae and adult flies.

Eighteen *UAS-RNAi* lines were also established. Insertions were mapped to the second or third chromosomes. Both *UAS-RNAi-NS2.1* and *UAS-RNAi-NS2.7* lines were used in this study; *UAS-RNAi-NS2.1* maps to the second chromosome, and *UAS-RNAi-NS2.7* maps to the third chromosome. Both lines are homozygous viable and fertile.

Antibody production and immunoblot analysis

For antibody production, the full-length *Drosophila NS2* cDNA (*SD10213*) in *pENTR* (see above) was inserted into *pDEST17* (Invitrogen) by Topo-mediated recombination. NS2 was expressed as an N-terminal 6XHis tag fusion protein in BL21 (DE3) *E. coli* cells by adding isopropyl β -D-1-thiogalactopyranoside (IPTG)

to 1 mM. We used a Ni⁺ affinity column as described by Novagen to purify the protein, and Aves Labs (Tigard, OR) prepared a chicken polyclonal antibody against the purified NS2.

Standard SDS-PAGE was used to resolve proteins from whole lysates from cultured embryonic Schneider 2 (S2) cells, as well as from larvae and adults transgenic for *UAS-GFP-NS2.2*. The proteins were blotted to nitrocellulose for 40 minutes using a semidry system (Bio-Rad Laboratories). Blots were blocked for 1 h in 3% nonfat dry milk that had been reconstituted in 0.9% NaCl (wt/vol), 100 mM Tris, pH 7.4, and 0.1% Tween 20 (TTBS). Blots were probed with the chicken polyclonal antibody described above diluted to 1/1000 in TTBS. The rabbit polyclonal antibodies specific for the carboxyl terminus of RpL23a or the central region of RpL34 were purchased from Abgent (catalog numbers AP1939b and AP13207c, respectively). Secondary antibodies include an affinity-purified, peroxidase-conjugated goat anti-chicken IgY (Pierce Chemical) diluted 1/250 with TTBS, and a peroxidase-conjugated goat anti-rabbit IgG (Promega) diluted 1/50,000 in TTBS. For analyses of both RpL23a and RpL34, three independent immunoblot experiments were performed, and representative results are presented.

RT-PCR

Total RNA was extracted from control and RNAi-expressing larvae using TRIzol® (Invitrogen) according to the manufacturer's recommendations. RNA concentrations were determined spectrophotometrically at 260 nm (NanoDrop Technologies). First strand cDNA synthesis was performed using SuperScript III

reverse transcriptase (Invitrogen) according to the manufacturer's recommendations with oligo-(dT). Primer sets for RT-PCRs were as follows: *Actin-5C* forward and reverse primers were 5'-CTCACCTATAGAAGACGAAGAAGTTGCTGCTCT-3' and 5'-CTAACTGTTGAATCCTCGTAGGACTTCTCCAACG-3', respectively; *NS2 (CG6501)* forward and reverse primers were 5'-CACCATGCCAAAGGTACGCAGCAC-3' and 5'-GCCTAGAGTGTAACGTAATATTTTACGCGTCCTTC-3', respectively. Thirty cycles were used in the RT-PCR reactions. Three independent RT-PCR experiments were performed and representative results are presented.

Immunofluorescence microscopy

Tissues from staged larvae were prepared for immunofluorescence microscopy as described (de Cuevas et al., 1996). In brief, tissues were fixed by buffered 2% formaldehyde for 0.5 h, washed in 1 X phosphate buffered saline containing 0.1% Triton X-100 (PBST), and probed overnight with the chicken anti-NS2 antibody (described above) diluted 1/1000 in 1 X PBST containing 5% normal goat serum. Tissues were washed with 1 X PBST and re-probed with an AlexaFluor 488-conjugated goat anti-chicken (Invitrogen) diluted 1/250 for 4 h. Tissues were then washed and counterstained with 4-6-diamidino-2-phenylindole dihydrochloride (DAPI; Polysciences) at 1.0 µg/ml.

Exogenous GFP-NS2 was expressed in larvae and adults by crossing the transgenic *GFP-NS2.2* line with the *da-GAL4* driver line. Tissues were fixed in a formaldehyde-containing buffer as described previously (Cui and DiMario, 2007)

before fluorescence microscopy. We used a Zeiss Axioskop equipped with a SPOT SE digital camera and software (Diagnostic Instruments) for conventional fluorescence microscopy.

Transmission electron microscopy

NS2-RNAi.1 and control larval tissues were fixed in TEM-grade 2% glutaraldehyde and 1% formaldehyde (Electron Microscopy Sciences) in 0.1 M cacodylate buffer (pH 7.0) for 1 h, and then rinsed overnight in 0.1 M cacodylate buffer (pH 7.0) containing 4 mM glycine. Tissues were post-fixed in 2% osmium tetroxide for 1 h, rinsed twice with water 5 min each, and then enblock stained with 0.5% uranyl acetate for 1 h in the dark. Tissues were dehydrated from 25% through 100% ethanol and embedded in LR White plastic. Thin sections were stained with Reynold's lead citrate and viewed with a JEOL JEM-1400 TEM operating at 120 kV. Images were captured with a Gatan UltraScan bottom-mount digital camera. All images were prepared for publication using Adobe's Photoshop CS6.

Results

NS2 homology

The *NS2* gene (*CG6501*) in *Drosophila* is located in cytological region 54C8-54C9 on the right arm of chromosome 2. Only one protein product, NS2, is predicted. NS2 contains 674 amino acids, and its mass is about 80 kDa by SDS-PAGE. Like all other MMR1_HSR1 GTP-binding proteins (Tsai, 2011), NS2 contains the well conserved circularly permuted GTP-binding motifs (G5', G4, G1, G2 and G3) (Figure 2.1). This is the most highly conserved region of the

HNS	MKK-----PKLK-KA---SKRMTCHKRYKIQKKVREHRRKLRKEAKKQGHK--KPRKDP--GVPNSAPFKEAL-----	60
NS1	MAL-----KRLKTKK---SKRLTGLKHKIEKKVRDHNKKERRAAKKNPKK--GSKKQKLQIPNICPFKDDI-----	63
NS2	MPKVRSTPGKPRTQG---FNHNSHSMNPERPKSGLKGVHAPRTKGTIKRLQMYRNFKAARDRTGKILTPAPFQGRRLPAGTMARVEPTPKW	87
Ngp1	MVK-----PKYKGRSTINPSKASTNPDBVQGA--GGQNMDRATIRRLNMYRQ--KERRNSRGKIIKPLQYQSTVASGTVARVEPNIKW	80
Consensus	M..K-----P..LK GK.---N-SKRSTNP..R..KI..KK..R..H..RK..RTIA...MY...--K...D...GKIPNPAPF...L---GT-ARVEP--KW	
Basic Region		
HNS	-----LREAELEKQR--LEELKQQ-----QKLDRO--KEQEKK-----RKLET--NPDIK	99
NS1	-----LKEVEEAKQROEAERLARR-----EAFKAE--REQNKF-----KTESMVEDADMR	107
NS2	FSNSRVISQTALOKFQDEIGKAVKDPYQVIMKPSQLPVTLLNEAAKYK--RVHLLDTESEFSTFGPKKQRRVSLKVRDLEDLSKAADDD	175
Ngp1	FGNTRVIGQSSLOKFGQEMDTVMKDPYKVMKQSKLPMSLLHDIRPHNLKVHILDTESFETTFGPKSQRRKPNLFAQDMQSLIENAEMS	170
Consensus	F-N-RV..K...ELQK...E..E..L..KDPY-V-MK-S-LP--LL-----VH-LDTEFDT.FGPKKEQRK--L--RDLES.L.ENADM.	
Coiled-Coil		
HNS	PSNVPEMEKEFGLOKCT--ENKAKSG--KNSKKLYCOELKKVIEASDVVLEVLDARDFLGCRCPQVEEAIQVS-GOKKLVLI LNKSDLV	183
NS1	STVHGIMHENDAQQD--EKKYKNAVTKEQSLKQYFKEFRKVIENADVLEVVDARDFLGTRCNEVERAVRGAPGNKRLVLVLNKKADLV	194
NS2	ADKYDSAKDDLIREDTGEKKAVRDWVFGAGQSKRIWNLHKVVDASVLLQVLDARDFMGTRSKYIEEFLRKEKPKHLLFFILNKVDLV	265
Ngp1	TESYDQGDRLDLYTETDGVREAEQEEIYKKGQSKRIWGLYKVIDSSDVVVQVLDARDFMGTRSPHILETYLKKKEPKWHILFVLLNKCGLV	260
Consensus	...YD..MKD..DL...EDTG-E..KAK...V..K..G..SKR..W..EL..KVI..ASDVVL.VLDARDF..GTR..P...EEALRKEK...KHLV...LNK..DLV	
G5'		
HNS	PKENLESWNLNKKELPTVVFRASTKPKDKGKIKRVKAKKNAAPFRSEVCFGKEGLWKLGGF--QETCSKATIRVGVIGFPNVGKSSI	271
NS1	PRENLNNWIKYFRSSGPTAFKASTQDQANRLGRRKLREMKTEKAMQGSVCIGAELLMSSMLGNYCNRKGIKTSIRVGVVIGFPNVGKSSI	284
NS2	PVWYTORWVAILSAEYPTIAFHASLQHP-----FGKGALINLFRQLGKLHLDKKQISVGFIGYPNVGKSSV	332
Ngp1	PTWATKRWVAVLSDYPTLAFHASLTNP-----FGKGAFIQLLRQFGKLHTDKKQISVGFIGYPNVGKSSV	327
Consensus	P...N...RWVAYLS.EYPT.AFHAS.Q.P-----K-----VCFGK..ALI..LL.QFGKLHTDKKQI..VG..IGYPNVGKSS..I	
G1		
HNS	NSLKQEQMCNVGVSMGLTRSMQVVPDLKQITIIDSPSFIVSPLNSSSALALRSPASIEVVK-PMEASAILSQADARQVVLKYTVPGYRN	360
NS1	NSLTRGRSMVGGSTPGVTSMQVEVLDISKIKLIDCPGIVFTSGGENSHAVLKNAQRVGVVDKPFITAESVLKRAKSEYFCTMYDITNYDT	374
NS2	NALRSKKVCKVPIAGETKVWQYITLMKRIFLIDCPGVVYPTAETDEKVLKGVVRVELVTNPEDYVDSLKKVRPEYISKNYKIEHWNT	422
Ngp1	NTLRSKKVENVAPIAGETKVWQYITLMRRIFLIDCPGVVYPSSEBETDVLKGVVQVEKIKSPEDHIGAVLERAKPEYISKTYKIDSWEN	417
Consensus	NSLRSKKVCNV..PIAGETK...QY..TL.KRIFLIDCPGVVYPS...S...VLKGVVRVE..VK..PED..A...VLKRA..PEYISK..YK...I....	
G2		
HNS	SLEFFFTMLAQRGMHOKGGIPNVEGAAKLLWSEWTGASLAYYCHP-----P-TSWTPPPYFNESI-----VMDMKSGFNLEELE	433
NS1	FEFFFAKKAARMGKFLKKGVPDVVAAARSVLNDWNTGKIKYCTQP-----PEVQEGQSVHISASI-----VHSEAREFDVNF	448
NS2	STHLEQLAQKTGKLLKGGEPDVTVTARMVLNDWQRGKLPFYVVP-----EGFAVPSQEGKEEEVVAE-----DPNEDAKSEAPTEVSESVK	505
Ngp1	AEDFLEKLAFTTGKLLKGGEPDLQTVGKMVLNDWQRGRIPEFVVPNAEPLVAPQLLPSSSLEVVPAAQNNPGEV-SETAGEGSESI	506
Consensus	SEEF..EKLQRTGKLLKGGEPDV...AA.MVLNDWQRGK..P..YV..P-----E-----P...QEG..S..EVVA..I-----P-E-V..TEA.G.F..SE.S.E	
Inhibitory Domain		
HNS	KNDQASIR-----AIKGPHLANSILFQSSGLTNGIIEEKDIHE-ELPK-----RKEERKQEEEREDDKSDOETADEE	498
NS1	SMETEILEHC-----AVK-----TDDIMEITIS--TGPLEIRQPRE-EAEPADKITATSLVIDEKEKPAKGRKRLDEEEKVDPS	519
NS2	KAR-----EFKQI-----QDFRKIRVGLLEYEQED--VKDLDIIDLELLEQ-----QKAERAAKKKARIHNLGEDEESS	567
Ngp1	KEETEENSHODANTEMQQLTRVRQNGFKINNVVPQFSGDDLVPVEVSDLEE-ELESFSD-----EEEEEQEQRRDDAEESSEPEEEN	588
Consensus	K..ETE...HC---E--QI--AVKQ..F..KI...Q..SG.D..GP..EVKDL..E-ELE...-----EKER..AK..R...DS...E..E..S	
Acidic Region		
HNS	V--DEN--SSGMFAAEETGEALSEETTAGEQSTR--SFILDKIIEEDDAYDFSTD-----	547
NS1	LLLEEN--QSLNKGIKQMOKLKKKQNVNRNEKKISKITDVLDSFSLGPSSSSKAEKYDFDED-----	577
NS2	DGADE--FYSEDEYNEDLQRVVHKKAKSKKPQQLAITSSGKFRV-AKIQPGSDGAGSSDDDGPS-----T SKAPR- L	636
Ngp1	VGNDTKAVIKALDEKIAKYQKFLDK-AKAKKFSAVRISKGLSEKIFAKPEEQRTLEEDVDDRAPSKKGGKKRKAQREEEQEHSNKAAPRAL	677
Consensus	VG..DEN---SLDE..IE...QK..L..K..AKAK...S...IT...L..SFILAKI..E..D...YDF...DD--PS-----KAPR-L	
HNS	-----YV-----	549
NS1	-----YV-----E	581
NS2	TAKQKRSLEERSQKRRKIGSNFYETTNVKNRNRNKKKD-----A	674
Ngp1	TSKERRRAVRQQRPKKVGVRYETHNVKNRNRNKKKTNDSEGQKHRRKKFRQK	731
Consensus	T-K--R--R-Q--KK-G---Y..T-NVKNRNRNKKK-----	

Figure 2.1 Protein sequence comparisons between human nucleostemin (HNS), *Drosophila* nucleostemin 1 (NS1), *Drosophila* nucleostemin 2 (NS2), and human Ngp1. NS2 shares 33% identity and 51% similarity with HNS. NS1 and NS2 share 33% identity and 49% similarity. *Drosophila* NS2 shares 48% identity and 65% similarity with human Ngp1, and is thus more closely related to Ngp1. Basic, coiled-coil, inhibitory, and acidic domains were initially identified and functionally characterized in NS (see text). The greatest conservations between the NS-like proteins occur within the permuted GTP bind domain that contains GTP-binding motifs G5', G4, G1, G2 and G3.

various NS-like proteins. NS2, however, also contains conserved basic, coiled-coil, inhibitory, and acidic domains that line up well with human NS (HNS) and *Drosophila* NS1 (Rosby et al., 2009). These auxiliary domains were first identified in rat NS (Tsai and McKay, 2002) and functionally characterized in several subsequent reports (Tsai and McKay, 2005; Bassler et al., 2006; Meng et al., 2006; Meng et al., 2007; reviewed by Tsai, 2011; Tsai, 2014)

NS2 shares 33% identity and 51% similarity with HNS, but 38% identity and 54% similarity with the related human GNL3L. NS1 and NS2 in *Drosophila* share 33% identity and 49% similarity, but unlike NS1 and HNS, NS2 contains additional residues downstream of the amino terminal basic domain, within the coiled-coil domain, and downstream of the acidic domain near the carboxyl terminus (Figure 2.1). Conversely, NS2 lacks residues as compared to HNS and NS1, most notably between G4 and G1. There is a 48% identity and 65% similarity between *Drosophila* NS2 and human Ngp1 with only minimal differences in amino acid insertions or deletions (Figure 2.1). Finally, a recent study by Matsuo et al. (2014) described *Drosophila* NS2 as an orthologue of yeast Nug2 (Ynr053cp, also known as Nog2); by our analysis, *Drosophila* NS2 shares 52% identity with Nug2 from *S. cerevisiae*, and 46% identity with Nug2 from the eukaryotic thermophile, *Chaetomium thermophilum*. Based upon these similarities, NS2 is orthologous to yeast Nug2 and human Ngp1 (Tsai, 2011, 2014).

Endogenous NS2 expression

We expressed His-tagged NS2 in *E. coli* and then purified the fusion protein using nickel affinity columns. A polyclonal chicken antibody was raised against this NS2 fusion. A previous antibody directed against NS1 (Rosby et al., 2009) labeled *E. coli*-expressed NS1 but failed to label *E. coli*-expressed NS2 (Figure 2.2, a). Likewise, the new antibody against NS2 labeled NS2 but failed to label NS1 (Figure 2.2, b). The chicken anti-NS2 antibody also labeled endogenous NS2 of ~80 kDa and the GFP-tagged NS2 protein at ~106 kDa in adult and larval lysates from a transgenic fly line that expressed GFP-tagged NS2 (Figure 2.2, c). The NS2 antibody also detected an endogenous NS2 in lysates of cultured embryonic S2 cells (Schneider, 1972). As a negative control, we show below that RNAi-depletion of NS2 eliminates the antigen labeled by this anti-NS2 serum (Figure 2.2, b). We conclude that the chicken anti-NS2 antibody is specific for NS2, and fails to label NS1.

Immunofluorescence labeling using the chicken anti-NS2 showed that endogenous NS2 is expressed in many different cell types in third instar larvae; Figure 2.3 shows just a few examples. NS2 was observed in the peripheral regions of nucleoli in midgut cells (Figure 2.3, a, b), diploid imaginal cells (Figure 2.3, c, d), tracheal cells (Figure 2.3, e, f), and fat body cells (Figure 2.3, g, h). The midgut, tracheal, and fat body cells have polyploid genomes, thus their nucleoli are relatively large, affording us the opportunity to locate NS2 readily to the peripheral regions of their nucleoli. We assume these NS2-positive peripheral regions are the granular components (GCs). The tracheal and fat body cells also

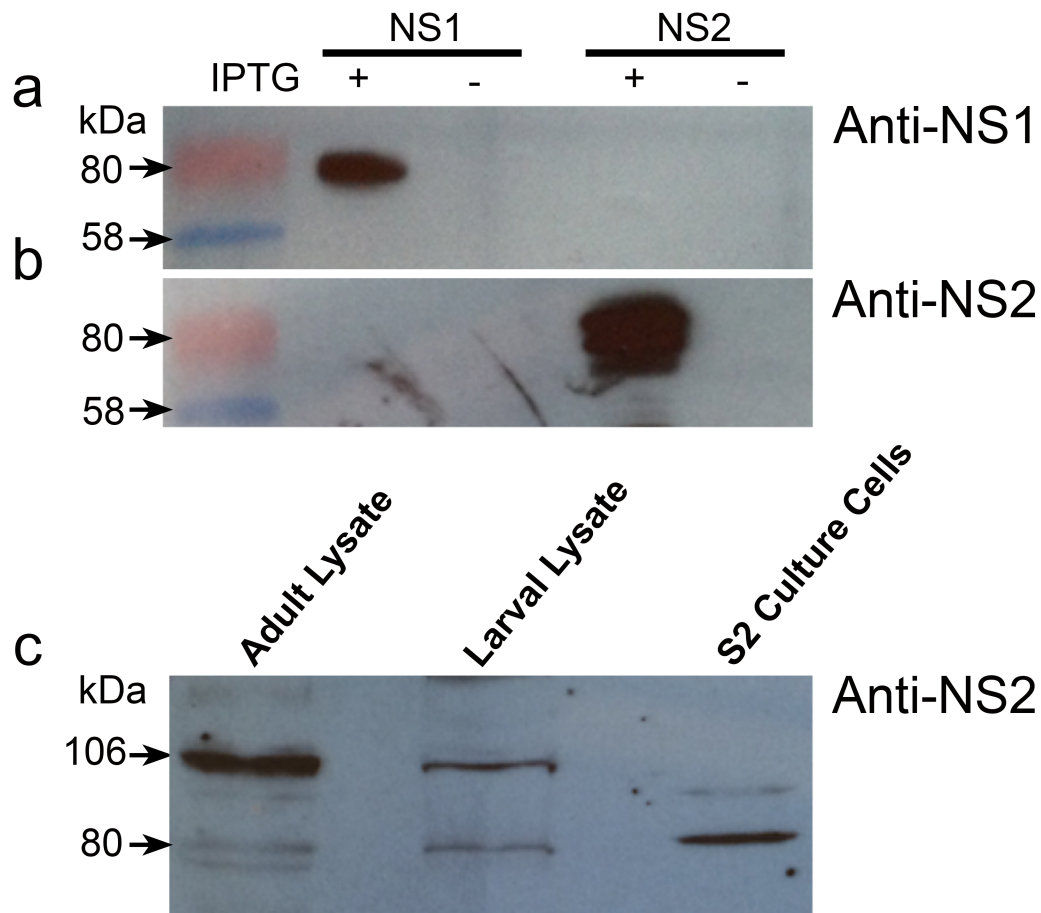


Figure 2.2 Immunoblots characterizing the specificity of antibodies directed against *Drosophila* NS1 and NS2. a-b Immunoblots containing lysates from *E. coli* cells that were incubated with (+) or without (-) IPTG that induces expression of His-tagged NS1 and NS2. a Immunoblot probed with anti-NS1 (from Rosby et al. 2009). The antibody labeled NS1 at 80 kDa, but failed to label NS2. b Immunoblot probed with anti-NS2. The antibody labeled NS2 and its modified versions, but failed to label NS1. c Immunoblot containing whole lysates from transgenic adult flies that expressed GFP-NS2 and endogenous NS2, transgenic larvae that expressed GFP-NS2 and endogenous NS2, or wild type S2 culture cells that expressed only endogenous NS2. The antibody labeled GFP-NS2 at 106 kDa and endogenous NS2 at 80 kDa.

showed additional antigenic sites dispersed throughout their nucleoplasm

(Figure 2.3, e, g). The cytoplasm of fat body cells also stained with the antibody

(Figure 2.3, g) under the fixation conditions used in this study.

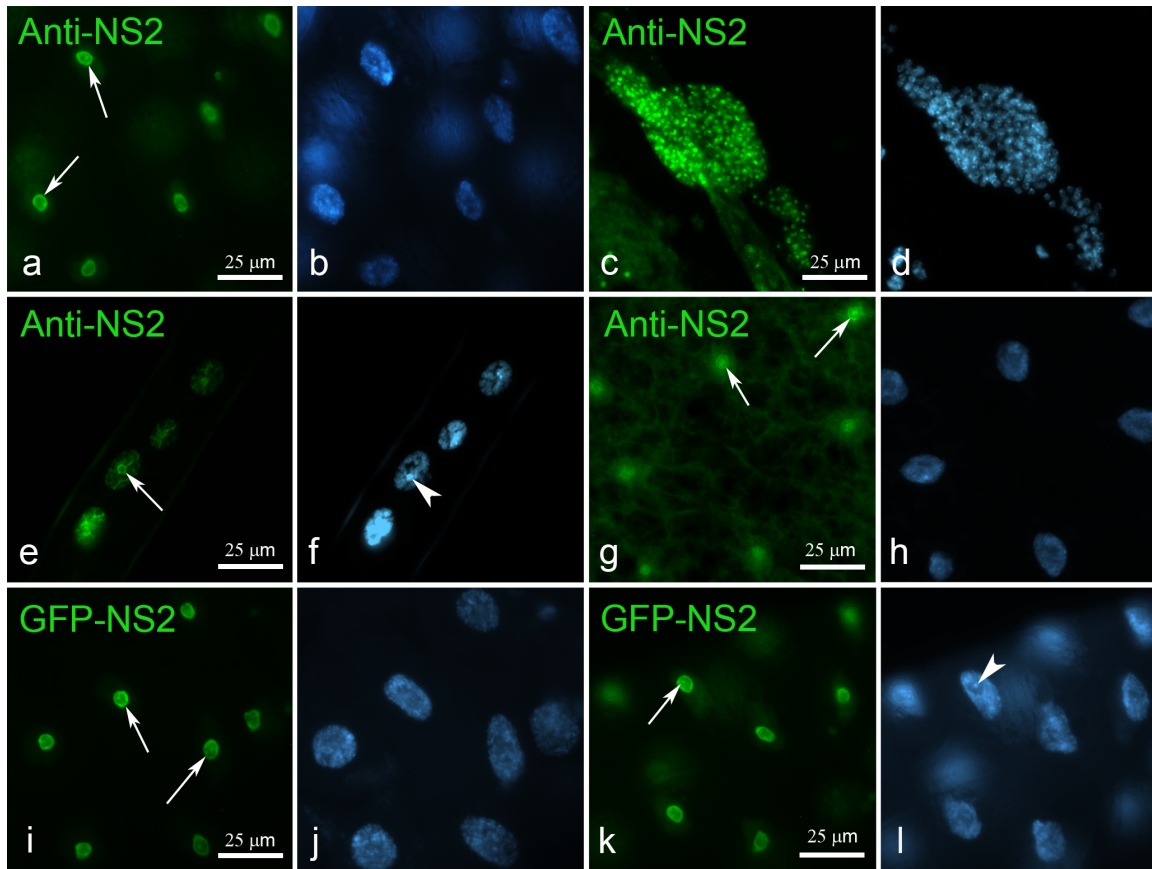


Figure 2.3 Immunofluorescence and GFP localization of *Drosophila* NS2 within various larval cell types. Immunofluorescence studies used the chicken anti-NS2. GFP studies used *da-GAL4 > UAS-GFP-NS2.1* progeny larvae. a-b Immunofluorescence and DAPI staining of larval polyploid midgut cells. The antibody preferentially labeled the peripheral components of the nucleoli (arrows). c-d Immunofluorescence and DAPI staining of smaller larval diploid cells. Nucleoli labeled well with the antibody. Bright DAPI spots within the nuclei are the heterochromatic chromocenters usually adjacent to the nucleoli. e-f Immunofluorescence and DAPI staining of larval tracheal cells. The antibody preferentially labeled the periphery of the nucleoli (arrow) and portions of the nucleoplasm. The DAPI-stained chromocenter is marked by an arrow head. g-h Immunofluorescence and DAPI staining of larval fat body cells. The antibody labeled peripheral and more centralized components of the nucleoli (arrows). In addition to the nucleoplasm, the antibody also labeled portions of the cytoplasm. i-j GFP-NS2 localized preferentially to the peripheral regions of nucleoli in larval midgut cells (arrows). Centralized bright spots were again present. k-l GFP-NS2 localized to the periphery and to a centralized spot (arrow) within the nucleoli of larval fat body cells. The DAPI-stained spot within the nucleolus (arrow head) did not co-localize with the spot containing GFP-NS2. Some GFP-NS2 was detectable in the nucleoplasm, but not in the cytoplasm.

Exogenous GFP-NS2 expression

To confirm NS2 subcellular locations, we expressed GFP-tagged NS2 using the GAL4-UAS system in *Drosophila* (*da-GAL4 > UAS-GFP-NS2.2*) (Brand and Perrimon, 1993). Just as with anti-NS2 labeling, GFP-tagged NS2 was detected in the peripheral regions of nucleoli in larval midgut cells (Figure 2.3, i, j). Interestingly, we observed endogenous NS2 and GFP-NS2 within the central regions of nucleoli in many, but not all of the midgut cells. Careful overlays indicated that these NS2-containing spots were not co-incident with DAPI-positive spots often found within the center of the nucleoli which we interpret to be the fibrillar centers (FCs). Ultrastructural images presented below show that GCs can normally occupy more centralized regions within these large nucleoli.

GFP-NS2 localization in midgut cells was essentially identical to the anti-NS2 labeling in the same cells. Unlike the antibody labeling, however, GFP-NS2 expressed in fat body (Figure 2.3, k, l) and tracheal cells (not shown) localized primarily to nucleoli with very little accumulation in the nucleoplasm or cytoplasm. While the antibody was reasonably specific for NS2 on immunoblots of larval lysates (Figure 2.2, c), and the antibody labeled nucleoli exclusively in larval midgut cells, the specific localization of GFP-NS2 to nucleoli in fat body cells indicated that the additional antigenic sites detected by the antibody in the nucleoplasm and cytoplasm of tracheal cells and fat body cells (Figure 2.3, e, g) may be spurious, at least under the fixation conditions used here.

NS2 depletion by RNAi

Two copies of the first 450 bp of the *NS2* cDNA coding sequence were ligated as inverted repeats downstream of five GAL4 *UAS* sequences in *pUAST*, a vector designed for fly transformation. We recovered 18 separate transgenic lines that expressed RNAi designed to deplete *NS2* mRNA. *UAS-NS2-RNAi.1* transgene maps to the second chromosome, and *UAS-NS2-RNAi.7* maps to the third chromosome. Both lines were used in these studies.

We crossed *UAS-RNAi-NS2.1* or *UAS-RNAi-NS2.7* lines with the homozygous *da-GAL4* driver line; the majority of NS2-depleted progeny from both crosses (*da-GAL4 > NS2-RNAi*) died in the second larval instar stage, but some could linger into the third instar. RT-PCR (Figure 2.4, a) showed a reduction in *NS2* transcripts in *da-GAL4 > UAS-RNAi-NS2.1* larvae compared to wild-type larvae, whereas *Actin5C* mRNA levels were unaffected. Immunoblot assays (Figure 2.4, b) using the chicken anti-NS2 described above showed that NS2 levels in *da-GAL4 > UAS-RNAi-NS2.1* larvae were also reduced compared to NS2 protein levels in wild-type larvae. The Actin5C protein served as a loading control. Immunofluorescence labeling using the same anti-NS2 showed that NS2 abundance in wild-type larval midgut cells was ample within nucleoli (Figure 2.4, c, panels 1 and 2), whereas labeling of midgut cells from *da-GAL4 > UAS-RNAi-NS2.1* larvae showed reduced amounts of labeling (Figure 2.4c, panels 3 and 4).

We previously showed that *Drosophila* NS1 is required for the release of the 60S subunit from nucleoli (Rosby et al., 2009). To test whether NS2 is also required for large ribosomal subunit release, we established larvae that

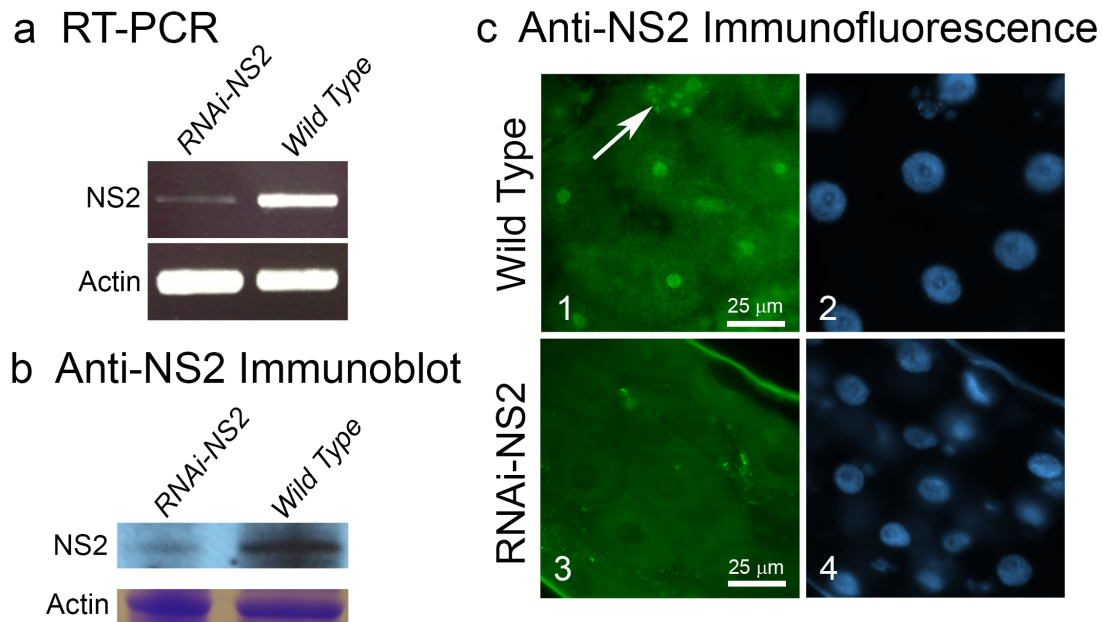


Figure 2.4 Assays demonstrating the depletion of NS2 by RNA interference. a RT-PCR showed the loss of *NS2* transcripts in larvae transgenic for an RNAi-expressing gene (*da-GAL4 > UAS-RNAi-NS2.1*) versus wild type larvae. *Actin* 5C transcripts in the same total RNA preparations served as controls. b Immunoblots containing total larval lysates were probed with the chicken anti-NS2 serum. Actin served as a loading control for the lysates. c Immunofluorescence with the chicken anti-NS2 serum and DAPI staining showed NS2 in nucleoli of wild type larval midgut cells (panels 1-2). Nucleoli in wild type midgut diploid imaginal island cells (arrow in panel 1) also labeled. Conversely, midgut cells expressing RNAi to deplete NS2 showed a loss of nucleolar labeling (panels 3-4).

expressed GFP-RpL11 either with normal NS2 levels (*da-GAL4 > UAS-GFP-RpL11*) or with NS2 depleted by RNAi expression (*da-GAL4 > UAS-GFP-RpL11; UAS-RNAi-NS2.7*). In the control larvae with normal NS2 levels, GFP-RpL11 accumulated in the cytoplasm of 2nd and 3rd instar midgut cells, presumably within large ribosomal subunits (Figure 2.5, a-d). GFP-RpL11 was barely detectable in the nucleoli of these cells, suggesting a minimum retention of GFP-RpL11 in nucleoli during normal ribosome biogenesis. These control larvae were

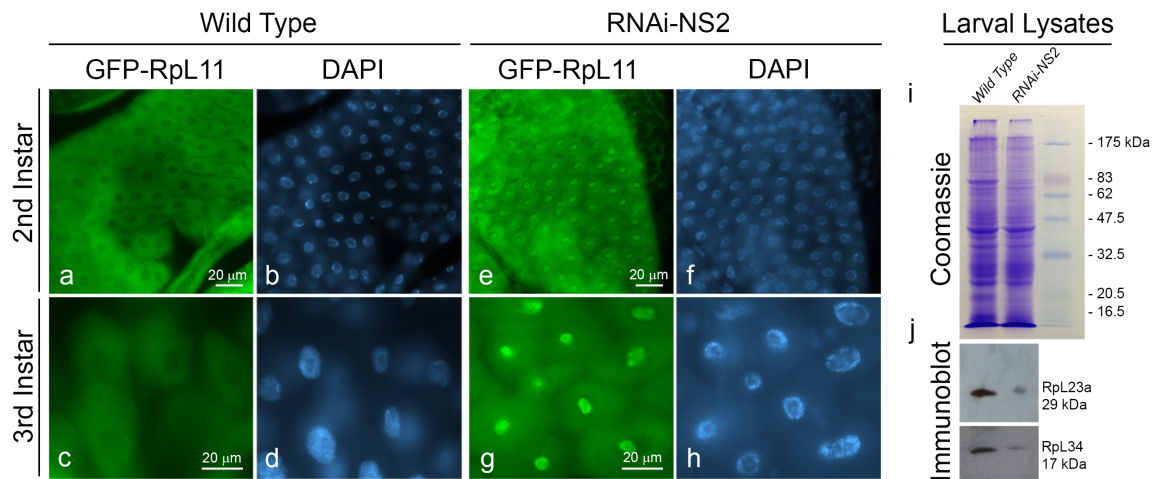


Figure 2.5 Loss of NS2 by RNA interference inhibited release of the 60S subunit as marked with GFP-RpL11 from nucleoli and lead to the loss of RpL23a and RpL34. a-d Midgut cells from second and third instar transgenic larvae but expressing NS2 (*da-GAL4 > UAS-GFP-RpL11; +*) showed preferential cytoplasmic labeling due to GFP-RpL11 incorporated into ribosomes. e-h Midgut cells from second and rare third instar transgenic larvae lacking NS2 (*da-GAL4 > UAS-GFP-RpL11; UAS-NS2 RNAi.7*) accumulated GFP-RpL11 within their nucleoli. i A Coomassie-stained SDS gel showing equal loads of whole larval lysates from wild type or NS2-depleted larvae. j Companion immunoblots were probed with anti-RpL23a or anti-RpL34. Reduced amounts of these proteins in the NS2-depleted larval lysates suggested an organism-wide loss of 60S subunits.

perfectly healthy, and all the larvae developed into adult flies, indicating that exogenous expression of GFP-RpL11 had no detrimental effects. In contrast, however, GFP-RpL11 accumulated within nucleoli when NS2 was depleted by RNAi (Figure 2.5, e-h). Similar to the depletion of NS1 (Rosby et al., 2009), there was no consistently observed accumulation of mRFP-RpS6 in nucleoli of NS2 depleted cells (not shown). All larvae depleted of NS2 died in the 2nd and 3rd larval instar stages.

With the 60S subunit blocked in its release from nucleoli in NS2-depleted cells, we wanted to determine if 60S subunit proteins were affected in terms of their abundance. By immunoblot analyses, both RpL23a and RpL34 showed declines in abundance in total lysates from NS2-depleted larvae compared to lysates from wild type larvae. Each respective immunoblot was repeated three times with essentially the same result. The observation suggests a general loss of 60S subunits. Thus we conclude that NS2 is required for the maturation or efficient release of the 60S subunit from the nucleolus to the nucleoplasm. The loss of RpL23a and RpL34 suggests an organism-wide loss of the 60S subunit.

Ultrastructural analysis of nucleoli

With the accumulation of GFP-RpL11 within nucleoli of NS2-depleted cells, we wanted to determine if these nucleoli had undergone any morphological changes at the ultrastructural level. Much of the nucleolar volume in wild type midgut larval cells consisted of the DFC with minimum amounts of the GC surrounding the DFCs on the periphery of the nucleoli (Figure 2.6, a). Small pockets of GC were typically found within the more centralized regions of these wild type nucleoli, perhaps explaining why the anti-NS2 antibody labeled internal nucleolar spots (see Figure 2.3, h, j above). Conversely, nucleoli in NS2-depleted larval midgut cells (Figure 2.6, b-d) contained copious amounts of GC, not only surrounding the nucleolus, but heavily infiltrating the DFC (Figure 2.6, b). Figure 2.6, c shows the GC of another nucleolus within a NS2-depleted cell; this GC extended far into the nucleoplasm. In addition to the extended GC, there were

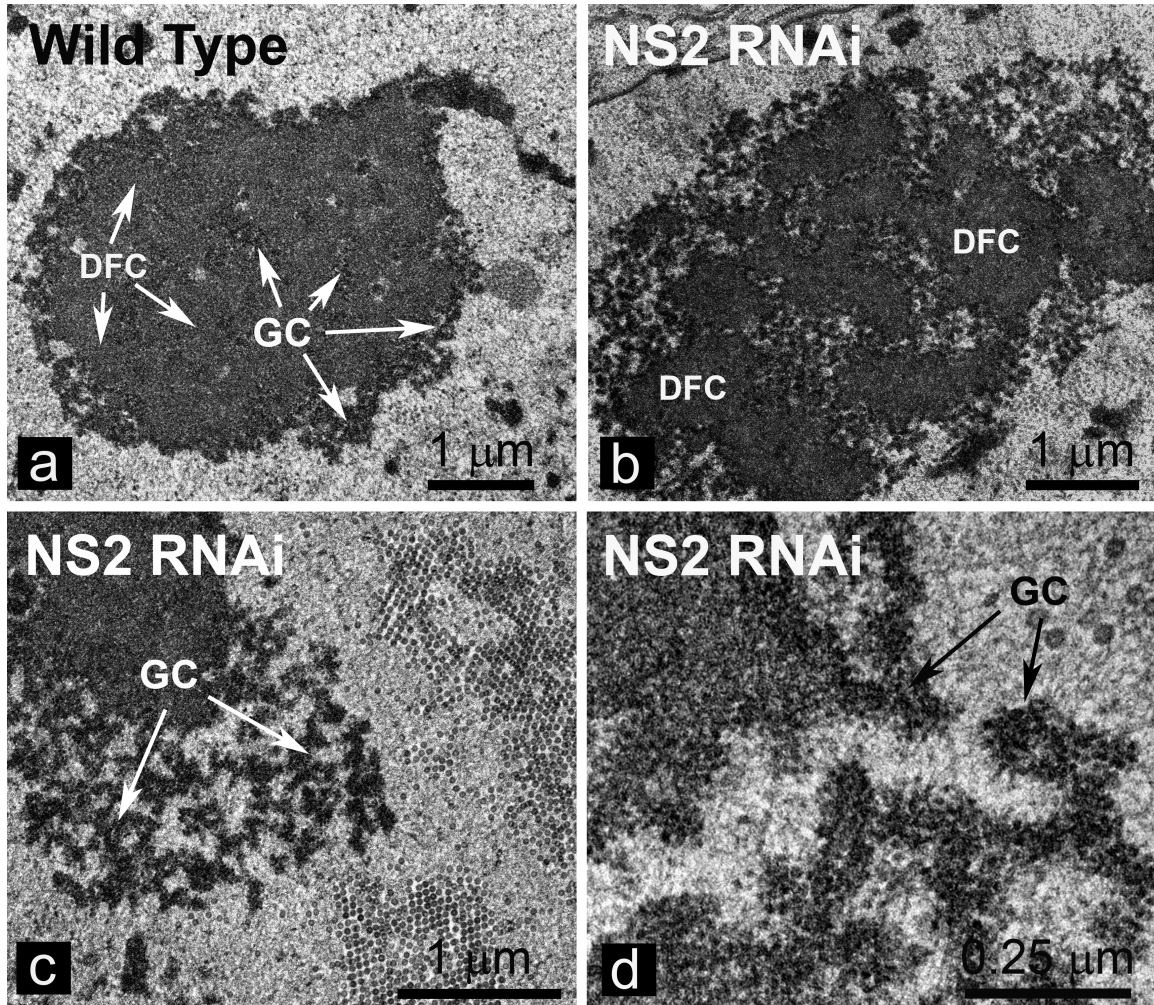


Figure 2.6 Ultrastructural analysis of nucleoli in larval midgut cells deficient for NS2. a Wild type cell with a normal nucleolus. Much of the nucleolus consists of DFC with GCs typically on the periphery of the nucleolus, but small pockets of GC are also evident within the nucleolus. b The nucleolus from a NS2-depleted cell displayed enhanced GCs not only on the periphery but within the interior of the nucleolus. c Some nucleoli displayed GCs that extended far into the nucleoplasm. The accumulation of excess spherical virus-like particles in the upper and lower right indicated the cell was under stress. d Higher magnification of the GCs that extended into the nucleoplasm. RNP granules were clearly evident with a few virus-like particles in the upper right.

many virus-like particles within the nucleoplasm of this cell. While a few of these particles are common to wild type nuclei, their quantity was typically high in the NS2-depleted nuclei, which served as an indication of cell stress (Strand and

McDonald, 1985; He et al., 2014). Granular RNPs were readily visible in extended GCs under slightly higher magnifications (Figure 2.6, d). The granules within the GCs were about half the size of cytoplasmic ribosomes, further indicating the 60S subunit was inhibited in its release from the GC.

In our previous studies on nucleolar stress in *Drosophila* larvae, we observed autophagy in polyploid gut tissues, but apoptosis in diploid imaginal tissues (Rosby et al., 2009; James et al., 2014). To demonstrate autophagy in NS2-depleted larval gut tissue, we again employed a *UAS-mCherry-Atg8a* transgene on the second chromosome either with or without the RNAi expressing transgene (*UAS-NS2-RNAi.7*) on the third chromosome. Midgut cells in well fed second instar larvae expressed mCherry-Atg8a (*da-GAL4 > UAS-mCherry-Atg8a*) with NS2 present, the autophagy marker diffused throughout the cells (Figure 2.7, a). TEM analysis of midgut cells from similar well-fed larvae showed copious amounts of cytosolic ribosomes, rER, and mitochondria (Figure 2.7, b) as expected. Other midgut cells, however, contained more or less rER depending on their physiological role along the alimentary canal (Martoja and Ballan-Dufrançais, 1984), and Figure 2.7, c shows an example of a midgut cell with less rER. With the loss of NS2 by RNAi expression (*da-GAL4 > UAS-mCherry-Atg8a; UAS-RNAi-NS2.7*), autophagy was clearly indicated by the accumulation of mCherry-Atg8a into cytoplasmic vesicles of the vast majority of midgut cells (Figure 2.7, d). TEM analysis of NS2-depleted midgut cells showed whorls of rER encircling mitochondria that were clearly degrading in terms of their internal morphology. Similar whorls of rER forming autophagic structures were described

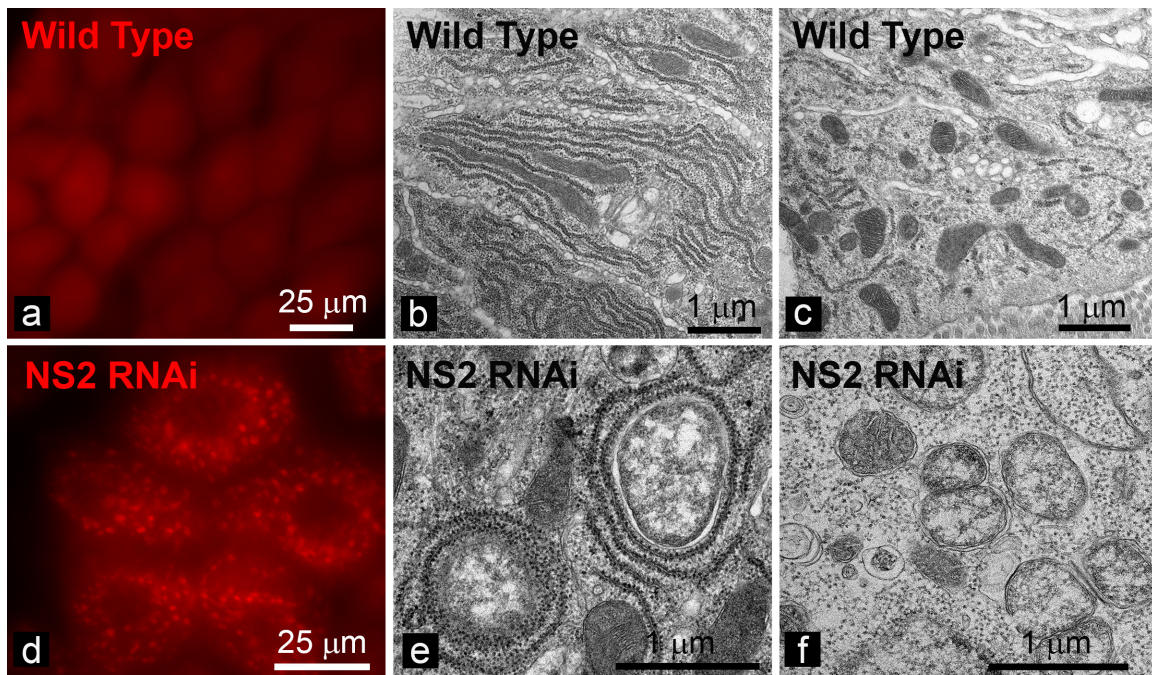


Figure 2.7 Depletion of NS2 induced autophagy in larval polyploid midgut cells. a Midgut cells from well fed wild type larvae that expressed mCherry-ATG8a (*da-GAL4 > UAS-mCherry-ATG8a*) showed a diffuse distribution of the marker protein. b Some wild type midgut cells showed copious amounts of rER and mitochondria. c Other wild type midgut cells showed less rER. Normal in-folding of the basal plasma membrane is in the upper right. Mitochondrial matrices in wild type cells were typically basophilic with cristae evident depending upon the orientation relative to sectioning. d Midgut cells that expressed mCherry-ATG8a but were depleted of NS2 (*da-GAL4 > UAS-mCherry-ATG8a; UAS-RNAi.7*) showed autophagic vesicles in the cytoplasm. e At the ultrastructural level, cells depleted of NS2 but rich in rER formed whorls of rER around autophagic vesicles that contained degrading mitochondria. f Cells depleted of NS2 but containing less rER continued to form autophagic vesicles that contained degrading mitochondria. The number of ribosomes in this cell was greatly diminished relative to normal midgut cells.

in *Drosophila* and rat (Helminen and Ericsson, 1971; Cormier et al., 2012). In midgut cells lacking substantial amounts of rER, degrading mitochondria were still present within autophagic vesicles (Figure 2.7, f). We believe the vesicles shown in Figure 2.7 (e and f) function as autolysosomes without ever acquiring

the electron-dense characteristic of classic lysosomes. As the NS2-depleted larvae aged, their midgut cells contained very few ribosomes as assayed by toluidine blue staining of 0.5 μm sections (not shown) (Feder and Wolf, 1965), but excessive numbers of autophagic vesicles (autolysosomes) and severely degraded mitochondria were apparent by TEM (not shown). We assume these polyploid cells die by continuous autophagy. Again, most of these larvae died in the second larval instar stage. None of the larvae reached the pupal stage. Overall, we conclude that loss of NS2 induces nucleolar stress, and at least the polyploid midgut cells responded to this stress by inducing autophagy to the point where the cells died.

Non-lethal depletion of NS2 in imaginal wing discs

To determine phenotypes due to NS2 depletion without inducing lethality, we used the larval wing disc-specific GAL4 driver, $P\{w^{+m*}=GAL4\}A9$ (referred to as *A9-GAL4*) on the X chromosome to induce *UAS-RNAi-NS2.1* on the second chromosome (*A9-GAL4 > UAS-RNAi-NS2.1*). We set up the cross such that all male progeny lacked the *A9-GAL4* driver and thus displayed normal wings (Figure 2.8, a). In contrast, all female progeny contained the *A9-GAL4* driver and expressed RNAi in their larval wing discs. While all the male progeny had normal wings, none of the *A9-GAL4 > UAS-RNAi-NS2.1* females displayed normal wings. The wing phenotype in these females varied from a relatively mild upward curl along the long edges of the wing to severely shriveled (vestigial-like) wings (Figure 2.8, a). Large fluid-filled blisters were common in the wings of newly eclosed females; their wings were left badly malformed as the blisters receded

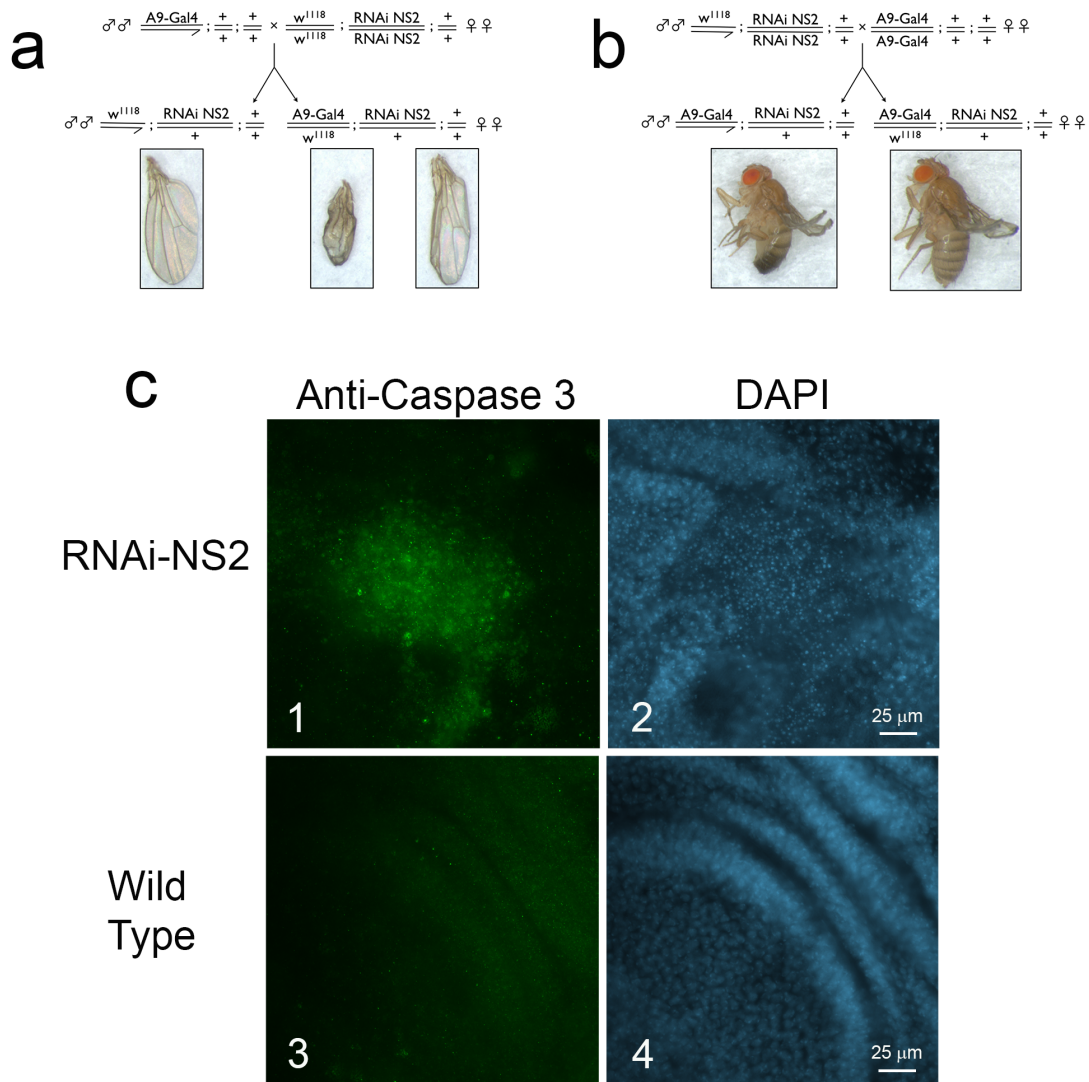


Figure 2.8 Depletion of NS2 in larval wing disc cells induced apoptosis. a Using the *A9-GAL4* driver on the *X* chromosome, a cross was set up to express RNA interference only in female progeny (*A9-GAL4* > *UAS-RNAi-NS2*. 1). Abnormal wing phenotypes (see text) were observed only in the female progeny. Male wings were normal. b A second genetic cross set up such that all progeny expressed RNAi in their wing disc cells. c Wing discs were removed from progeny larvae obtained from the cross in b. Wing discs from NS2-depleted larvae were probed with antibody directed against activated Caspase 3. Numerous apoptotic cells were evident. DAPI staining showed condensed DNA in the Caspase 3-stained cells (panels 1-2). Wild type wing discs served as a negative control (panels 3-4).

over time. When we reversed the cross (Figure 2.8, b) such that homozygous *UAS-RNAi-NS2.1* males were crossed to homozygous *A9-GAL4* driver females, all progeny adults (males and females) displayed badly malformed wings.

We previously showed that the loss of another nucleolar protein, Nopp140, in *Drosophila* wing discs induced apoptosis, and this led to malformed wings in adult flies (James et al., 2013). To test whether NS2 depletion also induced apoptosis, we used the cross in Figure 2.8, b to express NS2-RNAi in wing discs of all larval progeny. Anti-cleaved Caspase 3 (Asp175) from Cell Signaling Technology was used to detect apoptosis. This antibody is believed to detect the activated Caspase-9-like DRONC protein (Fan and Bergmann, 2010). A wing disc from an *A9-GAL4 > UAS-RNAi-NS2.1* larva was heavily labeled by anti-Caspase (Figure 2.8, c, panels 1 and 2), whereas the wing disc from wild type larvae (*w¹¹¹⁸*) showed minimal anti-Caspase labeling (Figure 2.8, c, panels 3 and 4). Therefore, selective loss of NS2 in *Drosophila* imaginal wing disc cells induced apoptosis, which likely explains the malformed adult wings.

Discussion

We showed that NS2 is expressed in most larval tissues. We focused primarily on the larval midgut for most of our studies as this tissue is easy to identify and isolate from small second instar larvae, and it offers various cell morphologies and physiologies (Filshie et al., 1971; Martoja and Ballan-Dufrançais, 1984). NS2 in midgut cells localized primarily in the nucleoli as seen by antibody and GFP labeling (Figure 2.3, a, h). Interestingly, Matsuo et al. (2011) described a shift in NS2 from the nucleolus to the nucleoplasm in larval

body wall muscle cells during larval development. Our antibody also labeled the nucleoplasm in larval tracheal and fat body cells (Figure 2.3, e, g). However, when we expressed GFP-NS2 in these cells, we saw robust nucleolar labeling in the fat body cells (Figure 2.3, j) and tracheal cells (not shown), but minimum GFP labeling in the nucleoplasm of these cells. Further analysis with the antibody under different fixation conditions and with fusion tags other than GFP should help resolve the discrepancy.

NS2 required for nucleolar release of the 60S subunit

We previously showed that depletion of *Drosophila* NS1 (GNL3) resulted in the accumulation of 60S subunits within nucleoli, with a corresponding reduction of cytoplasmic ribosomes as seen by TEM (Rosby et al., 2009). Like *Drosophila* NS1, yeast Nug1p and Nug2p/Nog2p (Yar053cp) belong to the same family of nuclear GTPases that display circular permuted GTP-binding domains. Bassler et al. (2001) showed that yeast Nug1p and Nug2p are separately required for the nuclear export of the pre-60S subunit; temperature-sensitive mutations in either gene inhibits 60S subunit export. They concluded that Nug1p and Nug2p were involved in late assembly/export of the 60S subunit. Saveanu et al. (2001) also showed that in the absence of Nog2p/Nug2p (Yar053cp), the large subunit leaves the nucleolus but accumulates in the nucleoplasm without being transported to the cytoplasm. Thus, the nucleolar GTPases Nug1p and Nug2p were predicted to function in the final maturation of the pre-60S subunit and/or its release to the nucleoplasm prior for export (Saveanu et al., 2003). Matsuo et al. (2014) strengthened this prediction by showing that the yeast

nucleolar GTPase, Nug2p, binds directly to the pre-60S subunit to block binding of export adaptor protein, Nmd3, to essentially the same site on the 60S subunit. In their model, potassium-dependent hydrolysis of the GTP bound to Nug2p releases Nug2p from the 60S particle allowing Nmd3 to bind and thus recruit nuclear export factors Crm1 and Ran-GTP. Matsuo et al. (2014) further demonstrated that mutations in Nug2p that block GTP hydrolysis resulted in 60S subunit accumulation within nucleoli. On the other hand, depletion of Nug2p allowed premature association of Nmd3 with the pre-60S subunit, but whether or not the 60S subunit was prematurely exported or retained within the nucleolus/nucleus upon Nug2 depletion was not described (Matsuo et al., 2014). Based on their observations, one might predict that the loss of NS2 (Nug2p in yeast) would result in premature export of the 60S subunit. Yet, in agreement with earlier reports (Bassler et al., 2001; Saveanu et al., 2001), we see the opposite result; namely, NS2 depletion causes GFP-RpL11 accumulation within expanded nucleolar GRs (Figure 2.6, b, c, d). Not only were the GCs extended into the nucleoplasm, but numerous GCs infiltrated the more centralized DFCs, perhaps explaining the appearance of GFP-RpL11 throughout the nucleolus by fluorescence microscopy. The earlier studies (Bassler et al., 2001; Saveanu et al., 2001) and our observations suggest that Nug2p and NS2 are required for release of the 60S subunit from the GCs. Thus its role seems to be more than just a 'placeholder' blocking Nmd3's binding the 60S subunit (Matsuo et al., 2014). Perhaps hydrolysis of NS2's GTP not only dissociates NS2 from the 60S subunit, but also causes a coordinated release of other non-ribosomal

processing factors still associated with the 60S subunit in the GCs. This combined release of several processing factors would liberate the 60S from the GCs, releasing it to the nucleoplasm for its eventual association with the export machinery.

Phenotypes associated with nucleolar stress

The loss of NS1 depleted cytoplasmic ribosomes as shown by TEM (Rosby et al., 2009). Here immunoblots of whole larval lysates showed that RpL23a and RpL34 levels were reduced in NS2-depleted larvae as compared to wild type (w^{1118}) larvae (Figure 2.5). These immunoblot assays measuring RpL23a and RpL34 were repeated three times each; all six efforts showed the same loss of these two proteins, suggesting that at least the 60S subunit may be degraded if not exported. Microscopic assays to confirm the loss of ribosomes in NS2-depleted tissues were mixed, however. While Figure 2.6, f shows a significant loss of cytoplasmic ribosomes, other TEM images showed near normal levels of cytoplasmic ribosomes despite the autophagy (Figure 2.6, e). This mixed result was supported by toluidine blue staining where some cells showed ribosome loss while other cells showed heavy cytoplasmic staining, even within the same tissue. We suspect, however, that overall ribosome loss was in general dependent on the age of the larvae.

Previous studies on NS1 depletion (Rosby et al., 2009) showed autophagy in hindgut cells (originally mis-identified as midgut cells). Autophagy was evident in these NS1-depleted hindgut cells as stacks of deeply invaginated plasma membrane encircled mitochondria to form autophagosomes and eventually

electron-dense lysosomes. Here we carefully kept track of the midgut through fixation, embedding, and thin sectioning, and note that autophagy is again evident upon nucleolar stress, but these midgut cells appear to use rER membrane as a source of inclusion membrane instead of the apparent plasma membrane as we saw in the hindgut cells. The different sources of inclusion membranes (Ravikumar et al., 2010; Puri et al., 2013) in midgut cells versus hindgut cells would suggest that cells use whatever membrane is most plentiful for autophagosome formation. Finally, classic electron-dense lysosomes were surprisingly under-represented in the midgut cells as compared to what we saw in the hindgut cells. We suspect that the autophagosomes in the midgut cells fused with endosomes to form amphisomes in which degradation occurred (Mizushima, 2007).

Loss of NS2 in the imaginal wing disc induced apoptosis as detected by anti-Caspase 3 labeling (Figure 2.8). Apoptosis rather than autophagy seems to be the preferred mode of response to nucleolar stress in the diploid progenitor larval tissues (imaginal discs). We observed identical results when we depleted the nucleolar chaperone, Nopp140, by RNAi expression (James et al., 2013).

Finally, NS1 and NS2 appear to have similar but non-complementary functions in the maturation or release of the 60S ribosomal subunit from the peripheral regions of nucleoli. Depletion of either protein is lethal, suggesting one cannot compensate for the loss of the other. While work on the yeast nucleolar GTPases clearly lead the field, we believe the polyploid tissues of *Drosophila* larvae provide sufficiently large nuclei and nucleoli to assess the roles of NS1

and NS2 in final 60S subunit maturation relative to the subunit's location within the nucleus. We hope to further define these functions in the metazoan, *Drosophila*.

Chapter 3

Deletion of *Drosophila* Nopp140 Disrupts Nuclear Homeostasis

Introduction

Nopp140 in metazoans (SRP40 in yeast) is a 140 kDa, highly phosphorylated protein (He and DiMario, 2011b). Nopp140 consists of three distinct domains: the amino terminal domain contains a LisH (Lis1-homology) dimerization motif (Kim et al., 2004), the large central domain that contains several alternating acidic and basic motifs, and finally a conserved carboxy domain (Meier, 1996).

Nopp140 is believed to function as a chaperone for snoRNP assembly and/or transport, and its functions are largely inferred from its interactions and co-localizations with other nucleolar and nuclear proteins. Studies report that Nopp140 co-localizes and interacts with several snoRNPs. For example, Nopp140 can co-precipitate with rat NAP57 (dyskerin in humans, Cbf5p in yeast), a pseudouridylase in box H/ACA snoRNPs (Meier and Blobel, 1994), and with fibrillarin, a methyltransferase in box C/D snoRNPs (Isaac et al., 1998; Yang et al., 2000). Nopp140 is required for the efficient localization of fibrillarin to the nucleolus and rRNA 2'-O-methylation (He et al., 2014). Nopp140 is likely a chaperone for shuttling snoRNPs between the nucleoli and Cajal bodies (CBs) (Isaac et al., 1998; Bellini and Gall, 1999). Besides snoRNPs, Nopp140 also co-localizes with p80 coilin in CBs (Vandelaer and Thiry, 1998) and interacts with the amino terminus of p80 coilin (Isaac et al., 1998), which is essential for organization of CBs (Liu et al., 2009). Nopp140 has been reported to interact with RNA Pol I (Chen et al., 1999; Lee et al., 2014) and rDNA (Tsai et al., 2008; Lee

et al., 2014), suggesting that Nopp140 might play a role in rDNA transcription. Overexpression of both full-length Nopp140 and a dominant negative Nopp140-carboxyl truncation resulted in Pol I mis-localization and changes in nucleolar structure (Chen et al., 1999).

Our previous work showed that loss of Nopp140 caused redistribution of fibrillarin to the nucleoplasm and impairment of 2'-O-methylation of 18S and 28S rRNA (He et al., 2014). Loss of Nopp140 also reduced the total amount of cytoplasmic ribosomes. To further explore the function of Nopp140, we examined the impact of Nopp140 deletion on three proteins: coilin, a CB component, and two nucleolar proteins, NS1 and NS2.

Materials and methods

Fly Stocks

Fly stocks were obtained from the Bloomington Stock Center at Indiana University unless otherwise noted. Lines used in this study included w^{1118} (Bloomington stock #3605) which we used as a wild-type control, the second chromosome balancer stock w^*/w^* ; *Sp1/CyO* originally from W. M. Saxton (Indiana University), the third chromosome balancer stock w^-/w^- ; *Scm^{Et50}e/TM3 Sb¹*, *Ser* originally from J. A. Simon (University of Minnesota) and abbreviated here simply as *TM3/Et⁵⁰*, the third chromosome balancer stock w^-/w^- ; *Sb/TM3*, *Ser*, *Act-GFP* (Bloomington stock #4534) abbreviated here as *TM3-GFP*, and the homozygous *daughterless-GAL4* driver line (Bloomington stock #55849). *P5CDh1^{f04633}/TM6* (*CG7145^{f04633}/TM6*, expressed simply as *WH-/TM6*) containing a *WH- pBac* element was originally obtained from the Exelixis collection at Harvard University. *Nopp140-/TM3-GFP* and *WH-/TM3-GFP* were

previously described (He and DiMario, 2011a; He et al., 2014). The homozygous *coil*¹⁹⁹ null mutation for the *coilin* gene was a kind gift from J. G. Gall (Carnegie Institution for Science) (Beumer et al., 2008). The *coil*^{199/199}; *Nopp140*-/TM3-GFP fly stock was constructed by crossing *coil*¹⁹⁹ and *Nopp140*-/TM3-GFP. All fly lines were maintained on standard fly food at room temperature (22-24° C).

Nopp140-/TM3-GFP were crossed inter se to generate homozygous *Nopp140*/- knockout progeny larvae that failed to fluoresce green compared to sibling larvae containing TM3-GFP (He et al., 2014). Homozygous *WH*/- and *coil*^{199/199}; *Nopp140*/- were hand selected. *WH*/- larvae were used in this study as controls to differentiate phenotypes caused by the *P5CDh1* gene disruption versus phenotypes caused by the *Nopp140* gene deletion.

Antibodies and immunoblot analysis

Standard SDS-PAGE was used to resolve proteins from whole lysates of larvae. The proteins were blotted to nitrocellulose for 40 minutes using a semidry system (Bio-Rad Laboratories). Blots were blocked for 1 h in 3% nonfat dry milk that had been reconstituted in 0.9% NaCl (wt/vol), 100 mM Tris, pH 7.4, and 0.1% Tween 20 (TTBS). The chicken polyclonal antibody for *Drosophila* NS1 was previously described (Rosby et al., 2009), and the chicken polyclonal antibody for *Drosophila* NS2 was described in Chapter 2. The guinea pig antibody against the amino-terminal fragment of coilin (GP1) was another kind gift from J. G. Gall (Carnegie Institution for Science) (Liu et al., 2009). Blots were probed with the chicken polyclonal antibodies for both NS1 and NS2 described above diluted to 1/1000 in TTBS. The coilin antibody was diluted to 1/10000 in TTBS. Secondary

antibodies included an affinity-purified, peroxidase-conjugated goat anti-chicken IgY (Pierce Chemical) diluted 1/250 with TTBS, and a peroxidase-conjugated goat anti-guinea pig IgG (Life technologies) diluted 1/5000 in TTBS. For all analyses, three independent immunoblot experiments were performed, and representative results are presented.

RT-PCR

Total RNA was extracted from control and RNAi-expressing larvae using TRIzol® (Invitrogen) according to the manufacturer's recommendations. RNA concentrations were determined spectrophotometrically at 260 nm (NanoDrop Technologies). First strand cDNA synthesis was performed using oligo-(dT) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. Primer sets for RT-PCRs were as follows: *Actin-5C* forward and reverse primers were 5'-CTCACCTATAGAAGACGAAGAAGTTGCTGCTCT-3' and 5'-CTAACTGTTGAATCCTCGTAGGACTTCTCCAACG-3', respectively; *Coilin* forward and reverse primers were 5'-GCACGACGAAGAATTACCAG-3' and 5'-GTGGCTACAATGATTTTGGC-3', respectively. Thirty cycles were used in the RT-PCR reactions. Three independent RT-PCR experiments were performed and representative results are presented.

Immunofluorescence microscopy

Tissues from staged larvae were prepared for immunofluorescence microscopy as described (de Cuevas et al., 1996). In brief, tissues were fixed by buffered 2% formaldehyde for 0.5 h, washed in 1 X phosphate buffered saline containing 0.1% Triton X-100 (PBST), and probed overnight with the chicken

anti-NS1 (1/1000), the NS2 antibody (1/1000) or the guinea pig anti-coilin (1/10000; described above) in 1 X PBST containing 5% normal goat serum. Tissues were washed with 1 X PBST and re-probed for 4 h with AlexaFluor 488-conjugated goat anti-chicken (Invitrogen) or AlexaFluor 546-conjugated goat anti-guinea pig (Molecular Probes) with both secondary antibodies diluted 1/250. Tissues were then washed and counterstained with 4-6-diamidino-2-phenylindole dihydrochloride (DAPI; Polysciences) or propidium iodide (PI) at 1.0 µg/ml.

We used a Zeiss Axioskop equipped with a SPOT SE digital camera and software (Diagnostic Instruments) for conventional fluorescence microscopy. We also used an inverted microscope (DMIRE2; Leica Microsystems, Deerfield, IL) equipped with TCS SP2 multiphoton hardware and software (Leica Microsystems) for confocal fluorescence microscopy.

Results

Endogenous Coilin Expression

P5CDh1 gene lies immediately downstream of *Nopp140*, but is transcribed in a reverse, convergent direction. Because the *pBac WH-* element used to delete the *Nopp140* gene lies within the 3' coding region of *P5CDh1*, the *P5CDh1* protein was truncated in *Nopp140*^{-/-} line. As a result, we used *WH*^{-/-} larvae, in which only the *P5CDh1* gene is disrupted by the *pBac* element, in all our experiments as controls to demonstrate that the phenotypes we observed in *Nopp140*^{-/-} were caused by loss of Nopp140 rather than the truncation of *P5CDh1*.

Drosophila coilin co-localizes with fibrillarin in Cajal bodies (CBs) (Liu et al., 2009). The same guinea pig antibody against coilin showed that endogenous coilin was expressed and located in the CBs in many different cell types in second instar larvae. Figure 3.1 shows that Nopp140-True and Nopp140-RGG localized to both nucleoli and CBs, and that coilin co-localized in the CB with the Nopp140 isoforms. Figure 3.2 shows examples of coilin localized to CBs in midgut cells (Figure 3.2, a, b), gastric caecum cells (Figure 3.2, c, d), and tracheal cells (Figure 3.2, e, f) of wild type larvae. Expression of endogenous

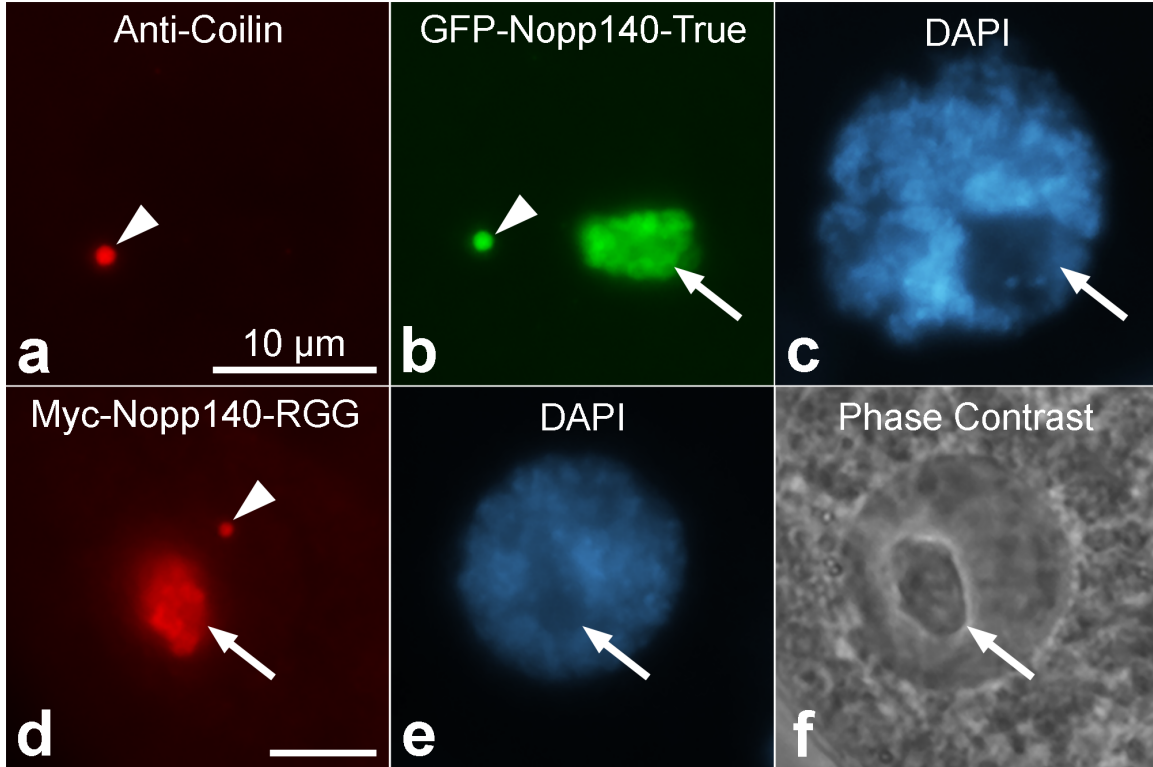


Figure 3.1 Localization of coilin and Nopp140. Nopp140-True (a-c) and Nopp140-RGG (d-f) localized in both the nucleoli and Cajal bodies (CBs). Anti-coilin labeled the CB (a, arrowhead), and coilin colocalized with Nopp140-True in the CB (b, arrowhead). GFP-Nopp140-True localized in both the nucleolus (arrow) and CB (arrowhead) (b). Myc-Nopp140-RGG showed Nopp140-RGG localized in both the nucleolus (arrow) and CB (arrowhead) (d). Nucleoli appeared as dark holes in DAPI stained nuclei (c, e); arrow in (f) shows the nucleolus by phase contrast microscopy. Bar, 10 µm for all images.

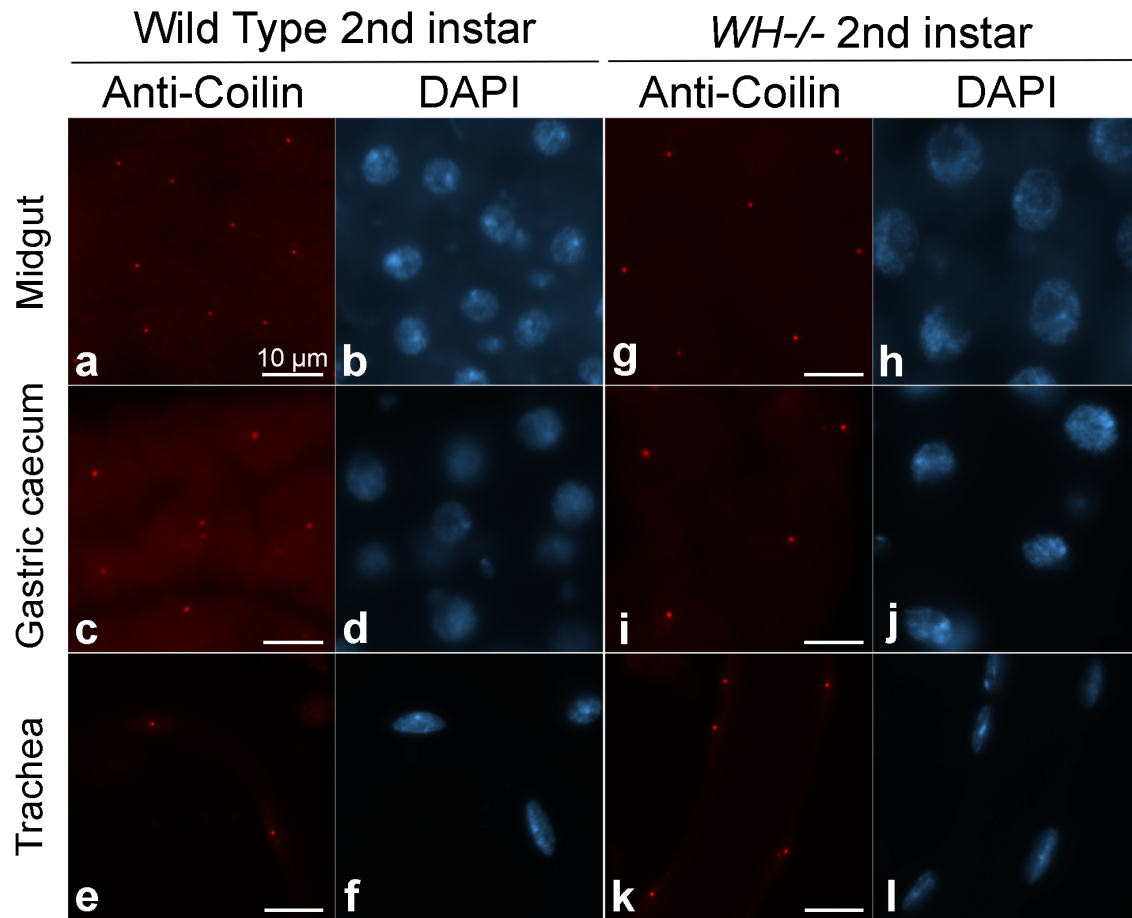


Figure 3.2 Immuno-labeling by anti-coilin in different cell types from wild type and *WH*^{-/-} larvae. All cells showed at least one CB labeled by anti-coilin. Midgut cells from wild type (*w*¹¹¹⁸) (a, b) and *WH*^{-/-} (g, h); gastric caecum cells from wild type (c, d) and *WH*^{-/-} (i, j); tracheal cells from wild type (e, f) and *WH*^{-/-} (k, l). There was no significant difference in fluorescent labeling between wild type cells and *WH*^{-/-} cells. Nucleoli appeared as dark holes in DAPI stained nuclei. Bar, 10 μ m for all images.

coilin in *WH*^{-/-} larvae was no different than that in the different wild type cells (midgut cells, Figure 3.2, g, h; gastric caecum cells, Figure 3. 2, i, j; tracheal cells, Figure 3.2, k, l). There was only one CB in most cell types in both wild type and *WH*^{-/-} larvae; few cells had more than one CB.

Loss of Nopp140 redistributed coilin from CBs, and elevated coilin protein level

Under normal conditions, coilin was concentrated in CBs in the nuclei of cells constituting the gastric caecum (Figure 3.3, e, f) and Malpighian tubules (Figure 3.3, g, h) in wild type second instar larvae. Surprisingly, coilin redistributed to the nucleoplasm in the same cells in *Nopp140*^{-/-} larvae (gastric caecum, Figure 3.3, a, b; Malpighian cells, Figure 3.3, c, d). However, we also noticed a high fluorescence background labeling in the cytoplasm in *Nopp140*^{-/-} cells. To confirm this phenotype in *Nopp140*^{-/-}, we constructed a *coil*^{199/199}, *Nopp140*^{-/-}*TM3-GFP* line. *coil*^{199/199} flies have coilin null mutation, thus there is no detectable coilin proteins in these flies (Beumer et al., 2008); but this coilin mutation is not lethal and these flies do not show reduction in viability or fertility.

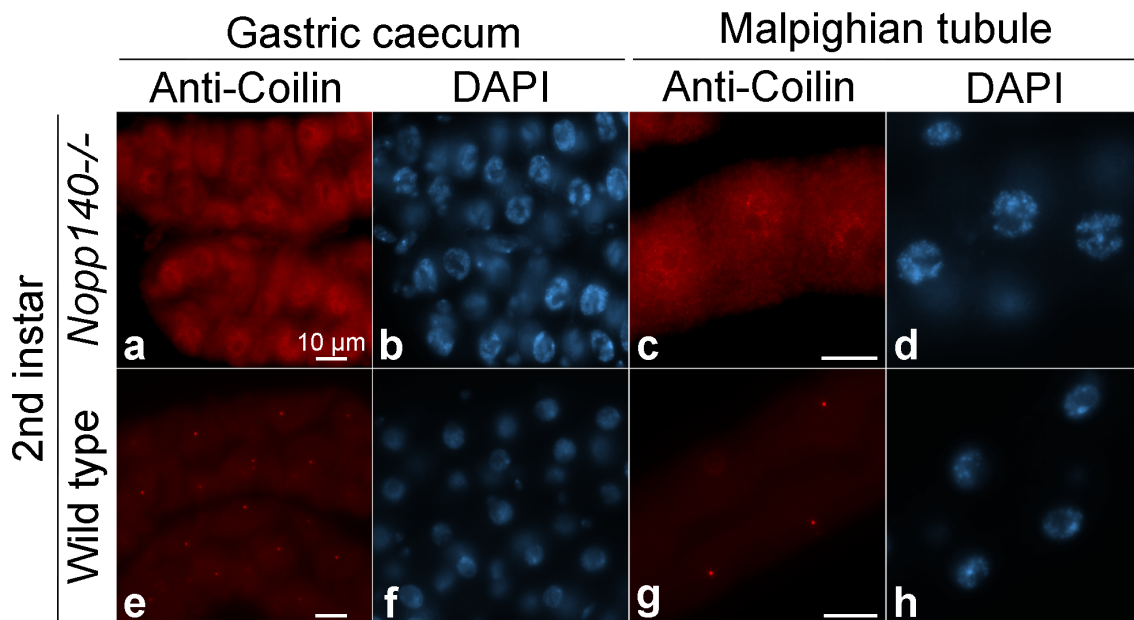


Figure 3.3 Knockout of *Nopp140* gene redistributed coilin from the CBs. Anti-coilin labeling and DAPI staining of gastric caecum cells from *Nopp140*^{-/-} (a, b) and wild type (e, f), or from Malpighian tubule cells from *Nopp140*^{-/-} (c, d) and wild type (g, h). Coilin redistributed from CBs to the nucleoplasm, and the coilin protein level appeared elevated in gastric caecum cells (a) and Malpighian tubule cells (c) compared to wild type cells (e, g). Nucleoli appeared as dark holes in the DAPI stained nuclei. Bar, 10 μ m for all images.

However, like homozygous *Nopp140*^{-/-} progeny, homozygous *coil*^{199/199}; *Nopp140*^{-/-} larvae were lethal in the second instar stage. Because *coil*^{199/199}; *Nopp140*^{-/-} larvae contained a null mutation at the *coilin* locus, they should not produce coilin. As we expected, we did not detect any labeled CBs in the nuclei of *coil*^{199/199}; *Nopp140*^{-/-} larvae. Figure 3.4 shows two samples, the gastric caecum (Figure 3.4, a, b) and the Malpighian tubule (Figure 3.4, c, d). However, we noticed a high background labeling in the cytoplasm again. To eliminate the possibility that loss of Nopp140 caused coilin redistribute into cytoplasm (Figure 3.3, a, c), we stained *coil*^{199/199}; *Nopp140*^{-/-} tissues using the same procedure, but only with goat anti guinea pig secondary antibody. We found that the

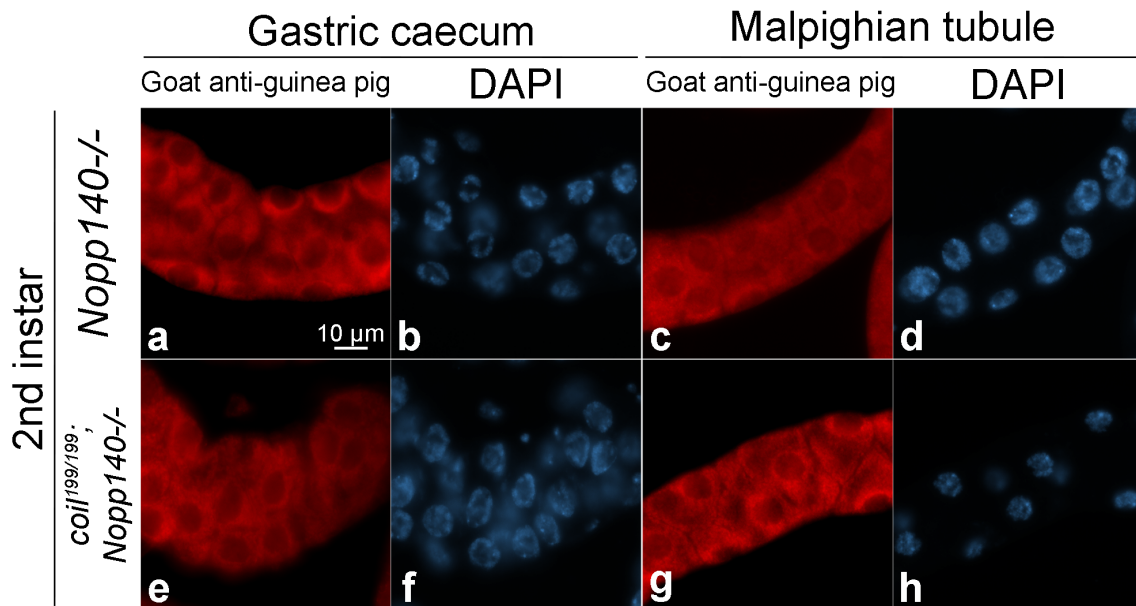


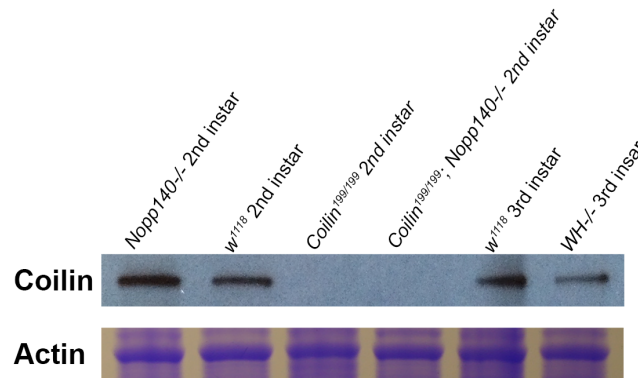
Figure 3.4 Secondary antibody test. AlexaFluor 546-conjugated goat anti-guinea pig (Molecular Probes) labeling and DAPI staining of gastric caecum cells from *Nopp140*^{-/-} (a, b), *coil*^{199/199}; *Nopp140*^{-/-} (e, f) and of Malpighian tubule cells from *Nopp140*^{-/-} (c, d), *coil*^{199/199}; *Nopp140*^{-/-} (g, h). The goat anti-guinea pig secondary antibody alone stained the cytoplasm of cells from *Nopp140*^{-/-} larvae (a, c, e, g). Nucleoli appeared as dark holes in DAPI stained nuclei. Bar, 10 μ m for all images.

secondary antibody itself labeled the cytoplasm (gastric caecum, Figure 3.4, e, f; Malpighian tubule, Figure 3.4, g, h). We also tried to stain tissues from wild type larvae by the secondary antibody, and we did not observe either nucleus or cytoplasm staining by the antibody. We do not know what the secondary antibody labeled in the cytoplasm in *Nopp140*^{-/-} tissues, but it did not affect our primary conclusion that loss of Nopp140 caused redistribution of coilin from the Cajal body to the nucleoplasm.

Full-length *Drosophila* coilin is ~90kDa on Western blots (Liu et al., 2009). We used the same anti-coilin antibody (GP1) for both immune-fluorescence microscopy and Western blots. The antibody labeled endogenous coilin at ~90 kDa in larval lysate from second instar *Nopp140*^{-/-} larvae, second instar *w*¹¹¹⁸ larvae, third instar *WH*^{-/-} larvae, and third instar *w*¹¹¹⁸ larvae (Figure 3.5, a). But no coilin was detected from second instar *coil*^{199/199} larvae or second instar *coil*^{199/199}; *Nopp140*^{-/-} larvae. We noticed that the relative abundance of coilin in 2nd instar *Nopp140*^{-/-} larvae was higher (about 2-fold) than that in second instar wild type larvae (Figure 3.5, a). A companion SDS-polyacrylamide gel showed that the actin band at 42 kDa was comparable in each sample, indicating equal loads of larval lysate. An earlier western blot showed ~10X excess coilin in *Nopp140*^{-/-} compared to wild type (not shown).

We next examined coilin mRNA expression level by RT-PCR. The coilin mRNA expression was elevated by about two fold in second instar *Nopp140*^{-/-} larvae compared to that in second instar wild type larvae (Figure 3.5, b). Actin mRNAs served as controls. As expected, we did not find any difference in coilin

a. Anti-Coilin Immunoblot



b. RT-PCR

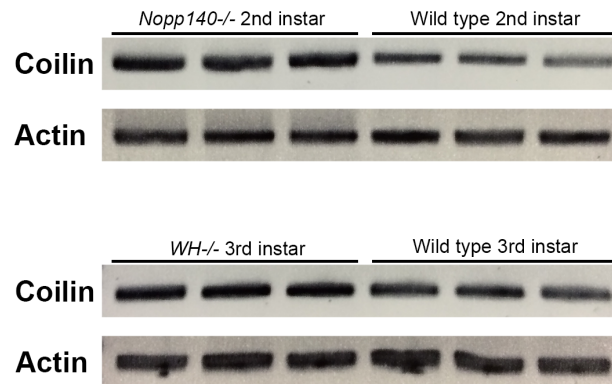


Figure 3.5 Loss of Nopp140 elevated coilin protein levels and mRNA levels. a Immunoblots containing total larval lysates were probed with the guinea pig anti-coilin antibody. The antibody labeled *Drosophila* coilin at ~90 kDa. Western blot showed coilin levels in Nopp140^{-/-} second instar larvae (the first lane) were elevated ~2-fold compared to wild type (the second lane). No coilin was detected in *coil*^{199/199} and *coil*^{199/199}; Nopp140^{-/-} larval extract (the third and fourth lanes). Actin at ~42 kDa on Coomassie-stained gel was used to determine equal loading of protein lysates. b RT-PCR showed the mRNA level was elevated in Nopp140^{-/-} second instar larvae. *Actin* 5C transcripts in the same total RNA preparations served as controls. Gel scanning and quantification showed the ratio of coilin mRNA levels to actin in Nopp140^{-/-} second instar larvae was ~2-fold compared to that in wild type second instar larvae.

protein levels and coilin mRNA expression levels between third instar *WH*^{-/-} and third instar *w*¹¹¹⁸ larvae.

Loss of Nopp140 caused redistribution of NS1 and NS2

To determine whether loss of Nopp140 also affects other nucleolar proteins besides fibrillarin (He et al., 2014), we tested how *Drosophila* NS1 and NS2 behaved after Nopp140 was knocked out. Immunofluorescence microscopy using chicken anti-NS1 antibody (Rosby et al., 2009) showed that NS1 enriched in the nucleoli of different cell types in both wild type and *WH*^{-/-} larvae. Figure 3.6 shows that NS1 was detected mainly in the nucleoli in midgut cells of third instar wild type (Figure 3.6, a, b) and *WH*^{-/-} larvae (Figure 3.6, c, d). Propidium iodide (PI) staining detected the nucleoli because of its high concentration of ribosomal RNA (Figure 3.6, b, d). We did not see any significant difference between wild type and *WH*^{-/-} cells. NS1 also located in the nucleoli in both midgut and gastric caecum cells of second instar wild type larvae (Figure 3.6, e, i), and again, PI mainly stained the nucleoli (Figure 3.6, f, j). However, in *Nopp140*^{-/-} cells, NS1 dispersed from the nucleoli to the nucleoplasm or cytoplasm (Figure 3.6, g, k). Loss of Nopp140 also affected rRNA, which redistributed from the nucleolus to nucleoplasm (Figure 3.6, h, l).

Drosophila NS2 is another *Drosophila* nucleostemin family protein that locates in the nucleolus of different cell types of third instar wild type (Figure 3.7, a) and *WH*^{-/-} larvae (Figure 3.7, c), and of second instar wild type larvae (Figure 3.7, d, f). Like NS1, NS2 redistributed from the nucleolus to nucleoplasm or cytoplasm upon Nopp140 depletion (Figure 3.7, g-i).

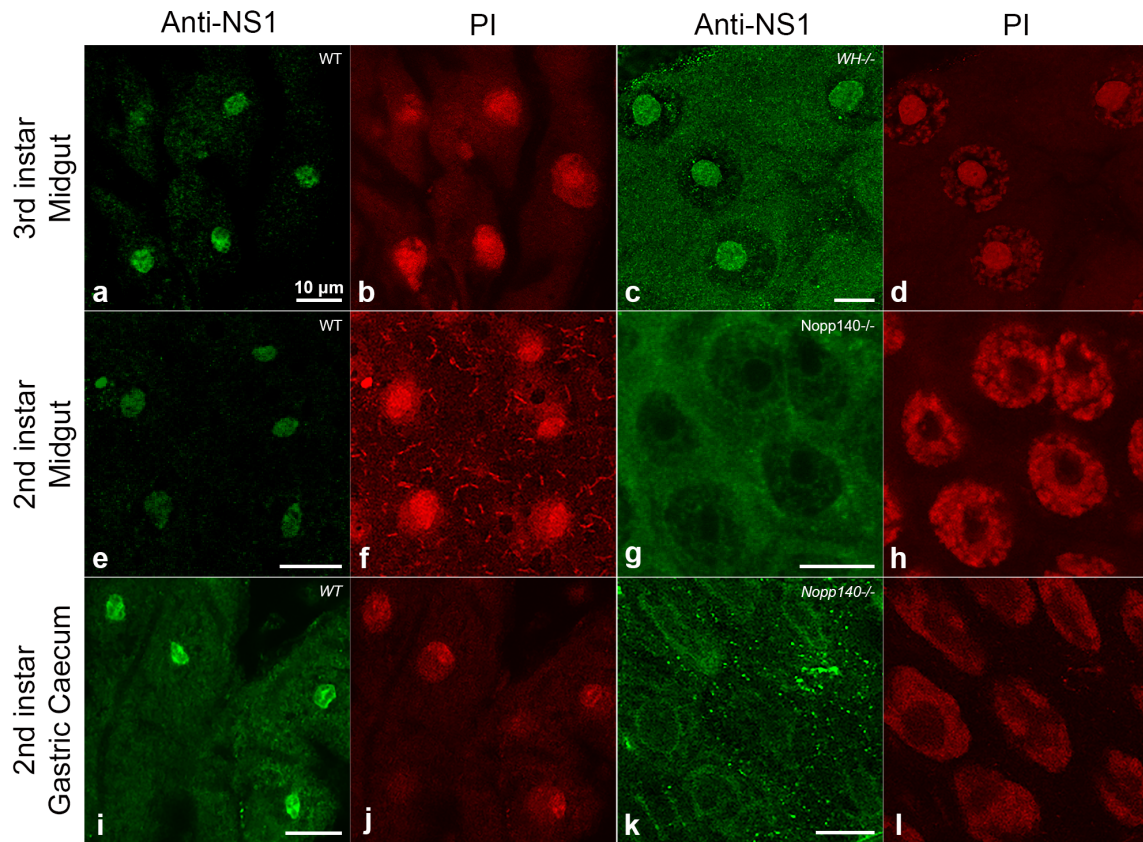


Figure 3.6 *Drosophila* NS1 redistributed from the nucleoli in *Nopp140*^{-/-} cells. Anti-NS1 labeling and PI staining of midgut cells from third instar wild type (a, b), *WH*^{-/-} (c, d), second instar wild type (e, f), *Nopp140*^{-/-} (g, h), and of gastric caecum cells from second instar wild type (i, j), *Nopp140*^{-/-} (k, l). NS1 mainly localized in the nucleoli in wild type and *WH*^{-/-} cells (a, c, e, i). PI stained mainly the nucleoli of wild type and *WH*^{-/-} cells (b, d, f, j). NS1 redistributed from the nucleoli to the nucleoplasm or cytoplasm in *Nopp140*^{-/-} midgut cells (g) and gastric caecum cells (k). rRNA stained by PI redistribute from the nucleoli and accumulated in the nucleoplasm in *Nopp140*^{-/-} midgut cells (h) and gastric caecum cells (i). Bar, 10 μ m for all images.

Discussion

We showed that loss of Nopp140 caused redistribution of coilin from Cajal bodies to the nucleoplasm. Besides redistribution, both microscopy and western blot analyses showed that coilin protein levels were increased by a minimum of two-fold in cells lacking Nopp140. Correspondingly, RT-PCR showed that the mRNA

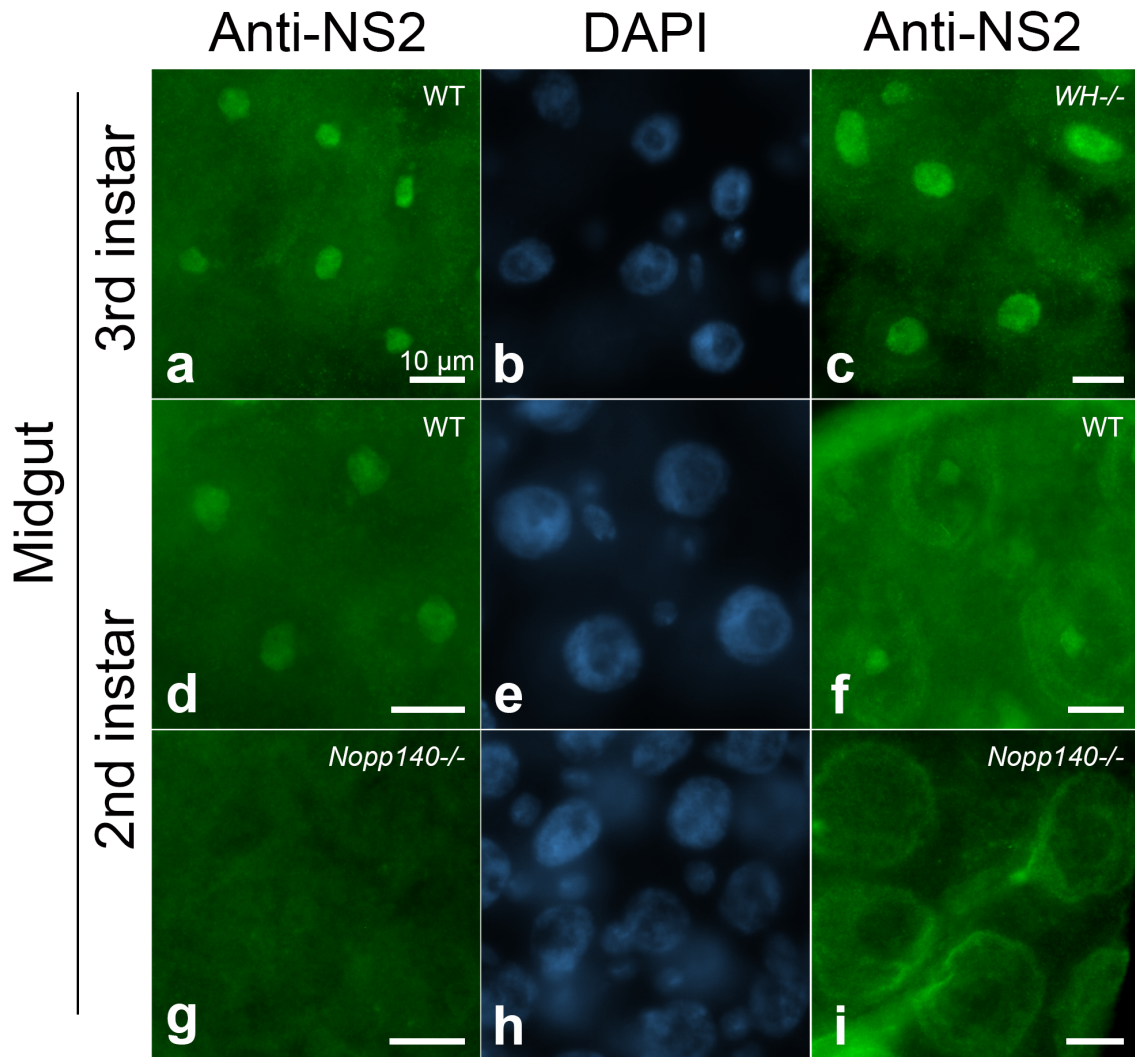


Figure 3.7 *Drosophila* NS2 redistributed from the nucleoli in *Nopp140*^{-/-} cells. Anti-NS2 labeling and DAPI staining of midgut cells from third instar wild type (a, b), *WH*^{-/-} (c), second instar wild type (d, e, f), *Nopp140*^{-/-} (g, h, i). NS2 was labeled in the nucleoli in wild type and *WH*^{-/-} cells (a, c, d, f). NS2 redistributed from the nucleoli in *Nopp140*^{-/-} midgut cells (g, i). Nucleoli appeared as dark holes in DAPI stained nuclei. Bar, 10 μ m for all images.

level of coilin was also elevated in the *Nopp140* knockout larvae by about two fold. Similar to coilin, two nucleolar proteins, *Drosophila* NS1 and NS2 also redistributed from the nucleolus to the nucleoplasm in the *Nopp140* knockout cells.

We previously reported that Nopp140 deletion led to redistribution of fibrillarin from the nucleolus to nucleoplasm (He et al., 2014). We also showed that 2'-O-methylation of 18S and 28S rRNA was inhibited in *Nopp140*^{-/-} cells, which may be caused by the redistribution of fibrillarin. However, loss of Nopp140 is not required for overall nucleolar integrity (He et al., 2014).

The Cajal body (CB) is a nuclear organelle present in all eukaryotes (Nizami et al., 2010). CBs are considered as a center of snRNA modification and spliceosomal snRNP (small nuclear ribonucleoprotein) assembly: small CB-associated RNAs (scaRNAs) and immature snRNP components including newly imported snRNP cores from the cytoplasm are all highly concentrated in CBs (Matera et al., 2009; Strzelecka et al., 2010; Novotny et al., 2011; Maticzka et al., 2014). Spliceosomal snRNPs visit CBs several times during their complicated maturation pathway (Filipowicz and Pogacic, 2002).

Besides snRNAs and scaRNAs, small nucleolar RNAs (snoRNAs) may be the most abundant class of RNAs within CBs, and all snoRNA classes concentrate in CBs (Machyna et al., 2014). CBs also contain the snoRNP protein fibrillarin and have a dynamic relationship with nucleoli (Gall, 2000; Platani et al., 2000). Nopp140, a chaperone for snoRNPs, shuttles between CBs and the nucleoli (He and DiMario, 2011b). Nopp140 can interact with amino terminus of p80 coilin, a widely accepted marker of CBs (Isaac et al., 1998) and these two proteins co-localize in CBs (Vandelaer and Thiry, 1998). Thus, CBs are also considered as the center of maturation and assembly of snoRNPs.

The p80 coilin protein is the most concentrated protein in CBs (Machyna et al., 2013). Coilin is essential for CB organization; CBs are disrupted in the absence of coilin (Liu et al., 2009). CBs act as catalysts that speed up snRNP assembly by concentrating necessary components (Klingauf et al., 2006; Novotny et al., 2011). Although cells of mice and flies lacking CBs due to coilin knockout have normal splicing levels (Tucker et al., 2001; Deryusheva and Gall, 2009), the retarded splicing can cause embryogenesis failure and fertility defects, such as increased in utero death and underdeveloped reproductive organs (Walker et al., 2009; Strzelecka et al., 2010). Since CBs also play an important role in snoRNP assembly, we assume that coilin glues all necessary factors together to increase the efficiency of snoRNP assembly. Here we showed that loss of Nopp140 caused redistribution of coilin, which led to an apparent loss of CBs in the nuclei. Since coilin is critical for the formation of the CB, we assume that *Nopp140* deletion also disrupts the integrity of CBs and its components, which decreases the efficiency of snRNP and snoRNP assembly. Interestingly, we discovered that coilin did not only redistributed, but the amount of coilin was elevated in larvae lacking Nopp140. Perhaps by producing more coilin in an attempt to provide more sites for RNP assembly, the cells tried to compensate for the lower efficiency of RNP assembly caused by CB disappearance. Perhaps both coilin and Nopp140 are required for the snoRNP assembly in CBs. Alternatively, Nopp140 may be required for coilin's "glue" function to maintain the integrity of CBs. Loss of one protein, Nopp140 in this case, forces the cell try to

make more the other protein, in this case coilin, to mitigate the loss of CB function.

Transmission electron microscopy (TEM) showed that loss of Nopp140 caused a tremendous decrease of ribosomes in the cytoplasm. Western blot supported the result by showing loss of Rpl23a and Rpl34 in larval lysates (James et al., 2013; He et al., 2014). We also showed that two nucleolar proteins, NS1 and NS2, are responsible for exporting large ribosomal subunits from the nucleolus. Depletion of NS1 led to accumulation of Rpl11 and Rpl26 within nucleoli, resulting in a loss of cytoplasmic ribosomes (Rosby et al., 2009). In addition, we showed in Chapter 2 that NS2 was responsible for maturation/exporting large ribosomal subunits, and that loss of NS2 caused reduced levels of Rpl23a and Rpl34. Since loss of Nopp140 displayed similar ribosome biogenesis phenotypes, we propose that ribosome biogenesis phenotypes caused by loss of Nopp140 may be partially due to the redistribution of NS1 and NS2 from the nucleolus. PI staining showed that rRNAs disappeared from the nucleoli, but they seemed to accumulate in the nucleoplasm. This result may indicate that NS1 and NS2 are important for exporting ribosomal subunits from the nucleus, and this function depends on proper nuclear homeostasis. However, Nopp140 has been reported to play a role in rDNA transcription (Chen et al., 1999; Lee et al., 2014). Redistribution of NS1 and NS2 may also be caused by the impaired rDNA transcription that leads to disruption of ribosomal biogenesis. Lack of proper ribosomal subunit biogenesis and assembly could cause the mis-location of NS1 and NS2.

Taken together, we conclude that Nopp140, working as a chaperon, is essential for the normal distribution of nucleolar and Cajal body proteins, and that these proteins depend on Nopp140 for their proper functions.

Chapter 4

Conclusions and Future Directions

Conclusions

The nucleolus and small GTPases involved in ribosome biogenesis

Although the nucleolus has been described as a multi-functional compartment in the nucleus, its main role is ribosome biogenesis. Almost all the nucleolar proteins are involved in ribosomal biogenesis. Chapter 1 reviewed the basic structure and function of the nucleolus focusing on ribosome biogenesis. During ribosome biogenesis, many GTPases play important roles in ribosome maturation and export. YlqF/YawG family GTPases are presented throughout all species and they share a very conserved structure—circularly permuted GTP binding motifs. Several GTPases in YlqF/YawG family (Nug1, Nog1, Nog2/Nug2 and Lsg1) are involved in ribosome biogenesis. Mammalian nucleostemin family proteins (NS, GNL3L, and Ngp1) belong to YlqF/YawG family. However, mammalian NS plays its role mainly in cell cycle progression and genomic DNA maintenance. In contrast, GNL3L and Ngp1 are more involved in ribosome biogenesis.

Drosophila NS2

Chapter 2 described *Drosophila* NS2 as orthologous to yeast Nug2 and human Ngp1. NS2 is expressed in all larval tissues examined. Like human Ngp1, NS2 in midgut cells localized primarily in the nucleoli as visualized by antibody and GFP labeling.

We previously showed that *Drosophila* NS1 (GNL3) plays an important role in exporting 60S subunits from the nucleoli. Loss of NS1 caused ribosomal

large subunits to accumulate within the nucleolus, with a corresponding reduction of cytoplasmic ribosomes as seen by TEM (Rosby et al., 2009). Like *Drosophila* NS1, yeast Nug1p and Nug2p/Nog2p (Yar053cp) belong to the same family of nuclear GTPases that display circular permuted GTP-binding domains. Yeast Nug1p and Nug2p (*Drosophila* NS2 ortholog in yeast) play different roles in late assembly/export of the 60S subunit (Bassler et al., 2001). Saveanu et al. (2001) showed that in the absence of Nog2p/Nug2p, the large subunits leave the nucleolus but accumulate in the nucleoplasm without being transported to the cytoplasm. Thus, the nucleolar GTPases Nug1p and Nug2p were predicted to function in the final maturation of the pre-60S subunit and/or its release to the nucleoplasm prior for export (Saveanu et al., 2003). A recent study further described the function of Nug2p in exporting the pre-60S subunit. Nug2p binds the same binding site of maturing nucleoplasmic pre-60S particles as that of Nmd3, a key pre-60S export adaptor (Matsuo et al., 2014). Nug2p binds the Nmd3 binding site on the ribosome and controls the proper time of recruitment of Nmd3 and other export factors (Crm1 and Ran-GTP) to the pre-60S subunit. Release of Nug2p from the LSU permits Nmd3 binding (Matsuo et al., 2014). The GTP-binding state of Nug2 is important for its binding to the pre-60S particle, and its GTP hydrolysis is crucial for its release from the position and the subsequent recruitment of Nmd3. Mutations in Nug2p that block GTP hydrolysis resulted in 60S subunit accumulation within nucleoli. On the other hand, depletion of Nug2p allowed premature association of Nmd3 with the pre-60S subunit (Matsuo et al.,

2014). However, whether or not the 60S subunit was prematurely exported or retained within the nucleolus/nucleus upon Nug2 depletion was not described.

Loss of NS2 (Nug2p in yeast) causes GFP-RpL11 accumulation within expanded nucleolar GRs, which agrees with earlier reports on Nug2 (Bassler et al., 2001; Saveanu et al., 2001). TEM microscopy supported the result by showing that the GCs not only extended into the nucleoplasm, but infiltrated the more centralized DFCs, which indicates ribosomal subunits accumulated in the nucleolus. The earlier studies (Bassler et al., 2001; Saveanu et al., 2001) and our observations suggest that Nug2p and NS2 are required for release of the 60S subunit from the GCs. Thus the role of NS2 in exporting 60S subunit seems to be more than just a 'placeholder' blocking Nmd3's binding site (Matsuo et al., 2014). If it were just a placeholder, loss of NS2 would have caused rapid release of LSUs.

Besides its role in the export of large ribosomal subunits, NS2 may play a role in exporting the small subunits. Our preliminary results from fluorescence microscopy showed that loss of NS2 also led to RFP-RpS6 accumulation in the nucleolus (Appendix A). In addition, the mass spectroscopy results showed that proteins co-precipitated with NS1 contains both 60S subunit proteins and 40S subunit proteins (Appendix A). We also found that an export factor Exportin-1 (i.e. Crm1) was pulled down with NS1, which supported our previous claim that NS1 is involved in the export of ribosomal large subunit (Rosby et al., 2009). NS1 and NS2 appear to have similar but non-complementary functions in the maturation or release of ribosomal subunits from the peripheral regions of nucleoli. Depletion

of either protein is lethal, suggesting one cannot compensate for the loss of the other.

The loss of NS1 depleted cytoplasmic ribosomes as shown by TEM (Rosby et al. 2009). In chapter 2 we showed that RpL23a and RpL34 levels were reduced in NS2-depleted larvae as compared to wild type (*w¹¹¹⁸*) larvae in immunoblot assays. However, results of both microscopic assays and toluidine blue staining to confirm the loss of ribosomes in NS2-depleted tissues were mixed. Some cells showed a significant loss of cytoplasmic ribosomes, others showed near normal levels of cytoplasmic ribosomes despite the autophagy. As a result, we suspect that overall ribosome loss was in general dependent on the age of the larvae, or differential expression of GAL4 and thus RNAi.

Loss of NS2 in the imaginal wing disc induced apoptosis as detected by anti-Caspase 3 labeling. Apoptosis rather than autophagy seems to be the preferred mode of response to nucleolar stress in the diploid progenitor larval tissues (imaginal discs). However, we observed autophagy in polyploidy gut cells lacking NS2. In addition, we observed the same pattern in other studies (Rosby et al., 2009; James et al., 2013). We suspect that cells use different mechanisms to respond to nucleolar stress.

Many studies have shown that mammalian nucleostemin plays an essential role in maintaining the continuous proliferation of cancer cells and stem cells (Tsai and McKay, 2002; Baddoo et al., 2003; Kafienah et al., 2006; Ma and Pederson, 2007; Dai et al., 2008; Meng et al., 2008; Ohmura et al., 2008; Qu and Bishop, 2012). Depletion of nucleostemin leads to cell cycle arrest at different cell

cycle stages, based on the types of tumor cells. In addition, most studies show that mammalian NS is involved in the cell cycle regulation, and its regulation is p53-dependent. However, Lin et al. (2014) suggested that nucleostemin and its paralog GNL3L (NS1 in *Drosophila melanogaster*, Nug1 in *Saccharomyces cerevisiae*, NST-1 in *Caenorhabditis elegans*, and Grn1 in *Schizosaccharomyces pombe*) share a common invertebrate ancestor. GNL3L however is closer to the common ancestor in terms of ribosome biogenesis than is nucleostemin. These two proteins started to diverge in function during the evolution of vertebrates: GNL3L is involved in the processing of pre-rRNA without DNA repair function (Lin et al., 2014) while nucleostemin has an essential role in maintaining the genomic integrity (Lin et al., 2013; Meng et al., 2013). In contrast to NS, the other two members of mammalian nucleostemin family, GNL3L and Ngp1 (NS2 in *Drosophila*, Nug2p in *Saccharomyces cerevisiae*,) have more direct invertebrate ancestors.

Drosophila Nopp140

Nopp140 is a highly phosphorylated nucleolar protein that is conserved among eukaryotes, from yeast to human (reviewed by He and DiMario, 2011b). The structure of Nopp140 contains conserved N-terminal and C-terminal domains, and a large central domain with alternating basic and acid regions (He and DiMario, 2011b). There are two Nopp140 isoforms in *Drosophila*, Nopp140-True and Nopp140-RGG, which share the same N-terminal and the large central domains, but differ in their C-terminal domains (Waggener and DiMario, 2002). Nopp140-True is homologous to mammalian Nopp140, while Nopp140-RGG

contains a distinctive Arg-Gly-Gly rich (RGG) carboxyl domain (Waggener and DiMario, 2002).

Nopp140 has multiple functions, and many of them are involved in ribosome biogenesis. Nopp140 plays an essential role in rDNA transcription. The hNopp140N382 protein (truncated from amino acids 1 to 382) mislocalizes the endogenous RNA polymerase I and shuts off rDNA transcription (Chen et al., 1999). Nopp140 interacts with both box H/ACA and box C/D small nucleolar ribonucleoproteins (snoRNPs), and functions as a chaperone for these snoRNPs which modify pre-rRNAs by pseudouridylation and 2'-O-methylation, respectively. Nopp140 can interact with NAP57, the pseudouridylase in box H/ACA snoRNPs (Lafontaine et al., 1998; Watkins et al., 1998). Nopp140 can also interact with NAP65 and fibrillarin, two components of box C/D snoRNPs (Watkins et al., 2004). We previously reported that the *Nopp140* deletion in *Drosophila* led to redistribution of fibrillarin from the nucleolus to the nucleoplasm (He et al., 2014). We also showed that 2'-O-methylation of 18S and 28S rRNA was inhibited in *Nopp140*^{-/-} cells, which may be caused by the redistribution of fibrillarin. However, loss of Nopp140 is not required for overall nucleolar integrity (He et al., 2014). Interestingly, the ribosomes that do appear in the cytoplasm tend to aggregate into ~40 nm clusters, suggesting they are defective in protein translation.

The data presented in this dissertation show that loss of Nopp140 caused redistribution of coilin from Cajal bodies to the nucleoplasm. Besides redistribution, this dissertation first reported that coilin protein levels were

elevated in the cells lacking Nopp140 by both immuno-fluorescence microscopy and western blot analyses. Correspondingly, RT-PCR showed that the mRNA level of coilin was also increased by about two fold in the Nopp140 knockout larvae. The coilin protein is the most concentrated protein in CBs (Machyna et al., 2013). The Cajal body (CB) is a nuclear organelle present in all eukaryotes (Nizami et al., 2010). CBs are considered a center for snRNA modification and spliceosomal snRNP (small nuclear ribonucleoprotein) assembly (Filipowicz and Pogacic, 2002; Matera et al., 2009; Strzelecka et al., 2010; Novotny et al., 2011; Maticzka et al., 2014). CBs are also considered as the center of maturation and assembly of snoRNPs (reviewed by Machyna et al., Nizami et al., 2010; 2013). Coilin is essential for organization of CB; CBs are disrupted in the absence of coilin (Liu et al., 2009). Coilin “glues” all CB components together (reviewed by Machyna et al., 2013), and CBs act as catalysts that speed up snRNP assembly by concentrating necessary components (Klingauf et al., 2006; Novotny et al., 2011). Nopp140 can interact with the amino terminus of coilin (Isaac et al., 1998), and these two proteins co-localize in CBs (Vandelaer and Thiry, 1998). Nopp140, a chaperone for snoRNPs, shuttles between CBs and the nucleoli (reviewed by He and DiMario, 2011b). Since coilin is critical for the formation of CB, we assume that the *Nopp140* deletion disrupts the integrity of CBs and its components, which reduces the efficiency of snRNP and snoRNP assembly.

Interestingly, we discovered that coilin did not only redistribute, but the amount of coilin was elevated in larvae lacking Nopp140. As far as we know, this is the first demonstration in which coilin gene expression has been affected.

Perhaps producing more coilin, the cells attempt to compensate for the lower efficiency of RNP assembly caused by the loss of CBs. Perhaps both coilin and Nopp140 are required for the snoRNP assembly in CBs; that is, Nopp140 is required for coilin's "glue" function to maintain the integrity of CBs. Loss of one protein, Nopp140 in this case, induces the cell to make more the other protein, coilin, to mitigate the defect.

Similar to fibrillarin, two other nucleolar proteins, *Drosophila* NS1 and NS2, redistributed from the nucleolus to the nucleoplasm or cytoplasm in the *Nopp140* knockout cells. Both NS1 (Rosby et al., 2009) and NS2 (Chapter 2) are responsible for exporting large ribosomal subunits from the nucleolus. Depletion of NS1 led to accumulation of RpL11 and RpL26 within nucleoli, and caused a loss of cytoplasmic ribosomes (Rosby et al., 2009). Chapter 2 described how the loss of NS2 caused RpL11 accumulation in the nucleolus and reduced the level of RpL23a and RpL34. In addition, we observed similar phenotypes in *Nopp140*^{-/-} larvae (He et al., 2014), and Nopp140 has been reported to play a role in rDNA transcription (Chen et al., 1999; Lee et al., 2014). Based on these results, we speculate that the redistribution of NS1 and NS2 may be caused by impaired rDNA transcription brought about by the depletion of Nopp140. With the loss of ribosomal subunits in the nucleolus, NS1 and NS2 redistribute from the nucleolus. Thus the adverse phenotypes caused by loss of Nopp140 may be partially due to the redistribution of NS1 and NS2 from the nucleolus.

Figure 4.1 summarizes the functions of Nopp140 in ribosome biogenesis. Under normal conditions, Nopp140 participates in several steps of ribosome

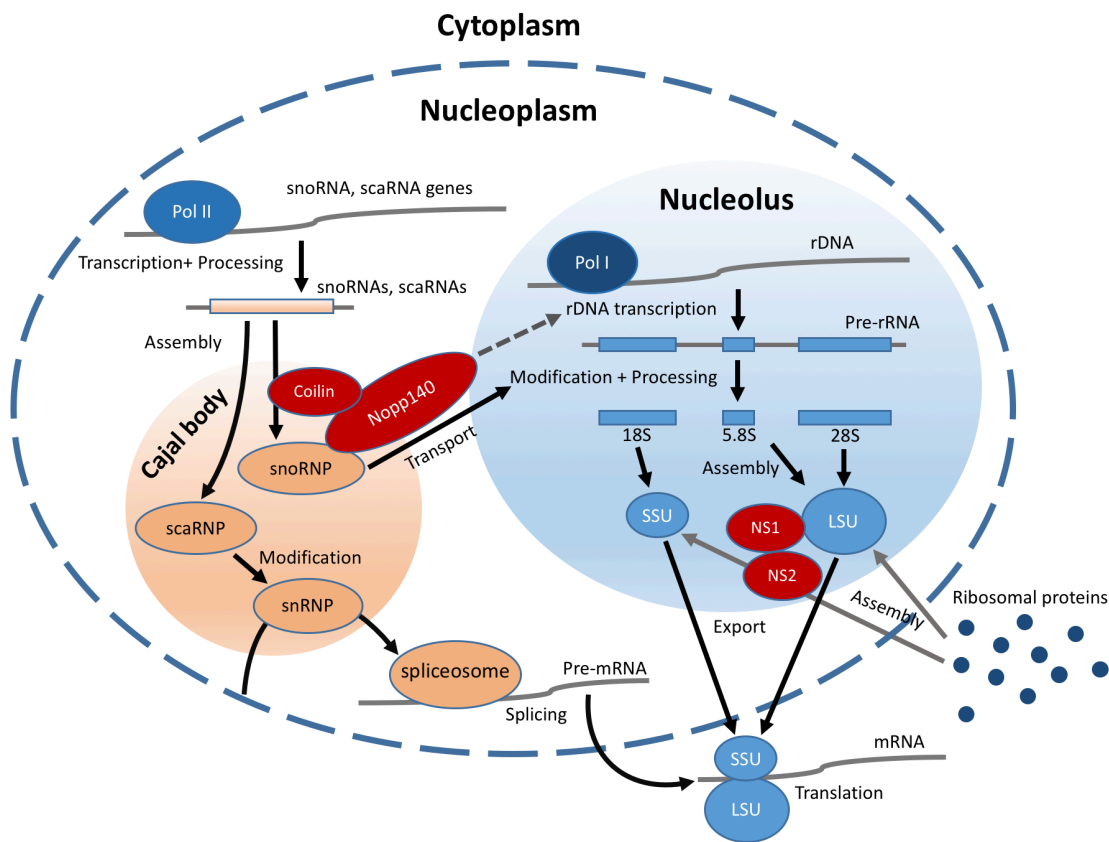


Figure 4.1 The roles of Nopp140 in nuclear homeostasis. The steps of ribosome biogenesis are shown from rDNA transcription by RNA Pol I to cytoplasmic assembly of small and large ribosomal subunits. Nopp140 plays important roles in rDNA transcription and pre-rRNA modification. The main functions of Cajal bodies are shown: assembly and modification of snRNPs and snoRNPs. Nopp140 functions as a chaperon to transfer snoRNPs from the CBs to the nucleolus. Nopp140 is also important for the formation of CBs. Loss of Nopp140 will cause the essential CB component, coilin to redistribute and lead to the disassembly of CBs.

biogenesis. First, Nopp140 is essential for rDNA transcription. In the absence of Nopp140, Pol I will mis-localize and rDNA transcription will be disrupted, which will lead to disruption of later steps in ribosome biogenesis. For example, if there is no properly assembled ribosome subunits, NS1 and NS2, two GTPases that are responsible for exporting ribosome subunits, will redistribute from the

nucleolus. Second, Nopp140 is involved in the modification and processing of pre-rRNA. Nopp140 functions as chaperone for snoRNPs, which pseudouridylate and 2'-O-methylate pre-rRNAs. Loss of Nopp140 causes redistribution of snoRNPs from the nucleolus, which leads to the disturbance of pre-rRNA modification and processing. Last but not least, Nopp140 also interacts and regulates coilin, a CB protein. Loss of Nopp140 may cause coilin up-regulation and redistribution of coilin, which leads to disassembly of CBs, and this then leads to lower efficiency in the maturation of snRNPs and snoRNPs. In summary, Nopp140 is important to maintain not only nucleolar homeostasis, nuclear homeostasis.

Future work

This dissertation described *Drosophila* NS2, which is homologous to the human nucleostemin-like protein, Ngp1. Like its paralog, *Drosophila* NS1, NS2 is involved in exporting ribosomal large subunits from the nucleolus. Preliminary data of fluorescence microscopy (Appendix A) showed that NS2 may also play a role in exporting small ribosomal subunits. In addition, immunoprecipitation experiments of NS1 (Appendix A) showed that NS1 interacted with not only ribosomal large subunit proteins, but also with small subunit proteins, which indicates that NS1 may also play a role in small subunits export. Although NS1 and NS2 share similar functions in ribosome biogenesis, depletion of either protein is lethal, suggesting that their functions are not complementary. Immunoprecipitation experiments of NS2 should provide further insights of its roles in ribosome biogenesis. Mammalian NS can bind to several proteins that

are involved in pre-rRNA processing, and it may play an essential role in pre-rRNA processing (Romanova et al., 2009). Immunoprecipitation experiments to identify binding partners of NS2 and sucrose gradient experiments to define NS2 complexes can help determine the role of NS2 in pre-rRNA processing.

Metabolic labeling to test the functionality of the ribosomes that remain in cells lacking NS2 could help in our understanding the impact on cells upon the loss of NS2.

In mammals, the *NS* gene is a direct transcriptional target of the oncoprotein, c-Myc, which activates gene expression by dimerization with its partner protein Max, followed by binding to the consensus DNA sequence CACGTG (E-box) (Zwolinska et al., 2012). Seventy percent of all human cancers are estimated to deregulate Myc oncoproteins. In mammals, c-Myc binds the E-box, which is ~60 bp upstream of the transcription start site (TSS) in the *NS* gene. Thus *NS* is downstream of c-Myc, and is regulated by c-Myc (Zwolinska et al., 2012). In *Drosophila*, ribosome biogenesis and ribosome protein genes are core dMyc targets, with 77% of these genes containing E-box binding sites for dMyc within 100 bp of their TSSs (Herter et al., 2015). Interestingly, *NS1* gene has an E-box at ~50 bp upstream of its TSS, and the *NS2* gene has an E-box at ~70 bp upstream of its TSS. This suggests that both genes may be targets of dMyc. However, whether *NS1* and *NS2* genes are dMyc targets, and whether NS1 and NS2 are part of dMyc pathway in regulating cell growth remain unknown.

Chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR)

experiments should help to answer these questions by using mutant lines in which the E-box have been disrupted.

Loss of NS2 in the imaginal wing disc induced apoptosis as indicated by anti-Caspase 3 labeling. The diploid progenitor larval tissues (imaginal discs) seem to prefer apoptosis rather than autophagy to respond to nucleolar stress. We observed identical results when we depleted the nucleolar chaperone, Nopp140 (James et al., 2013). Loss of Nopp140 activates c-Jun N-terminal Kinase (JNK), which induces apoptosis by activating the pro-apoptotic gene *Hid*. JNK may be a central stress response effector that is activated by nucleolar stress (James et al., 2013). RT-PCR and western blot analyses will help to determine what signaling pathways lead to apoptosis in the NS2-depleted cells, and to confirm whether JNK is the central stress response effector for nucleolar stress in *Drosophila*.

This dissertation further explored the impacts of loss of Nopp140 by showing that an essential CB protein, coilin, and two nucleolar proteins, NS1 and NS2 redistributed upon Nopp140 depletion. However, the exact mechanism of how Nopp140 causes distribution of these proteins is not clear. Partial depletion of Nopp140 by RNAi will help us better understand the function of Nopp140 in maintaining nucleolar and nuclear homeostasis. *Nopp140* knockout depletes both isoforms of Nopp140, Nopp140-True and Nopp140-RGG. The two isoforms may have different functions in ribosome biogenesis, so effort to express either Nopp140 isoform in the *Nopp140* gene knockout background will help determine function of individual isoforms. Our recent efforts showed that expression of

Nopp140-True alone in the *Nopp140* gene knockout was synthetic lethal at the embryonic stage, which leads to us to formulate a new hypothesis, namely that equimolar amounts of the two Nopp140 isoforms are important for nucleolar and nuclear function. Based on the results of expression of Nopp140-True alone, we assume that we should observe similar results from expression of Nopp140-RGG alone. If that is the case, a rescue experiment to express both isoforms of Nopp140 should help us to fully understand the function of Nopp140 in *Drosophila*.

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Appendix A

Extension Experiments and Results

In this appendix, we describe additional experiments that were performed to ascertain the functions of NS1 and NS2. Here we report preliminary findings; further work is needed to understand the implications of these results.

Materials and Methods

Fly stocks

Fly stocks were obtained from the Bloomington Stock Center at Indiana University unless otherwise noted. Lines used in this study included w^{1118} (Bloomington stock #3605) which we used as a wild-type control, the second chromosome balancer stock w^*/w^* ; *Sp1/CyO* originally from W. M. Saxton (Indiana University), the third chromosome balancer stock w^-/w^- ; *Scm^{Et50} e/TM3* *Sb¹*, *Ser* originally from J. A. Simon (University of Minnesota) and abbreviated here simply as *TM3/et⁵⁰*, the third chromosome balancer stock w^-/w^- ; *Sb/TM3*, *Ser*, *Act-GFP* (Bloomington stock #4534) abbreviated here as *TM3-GFP*, the homozygous *daughterless-GAL4* driver line (Bloomington stock #55849), and w^* ; *P{GAL4-ey.H}4-8/CyO*, abbreviated here as *ey-Gal4/CyO* that strongly expresses GAL4 in the eyeless pattern (stock #5535). Transgenic flies that express *UAS-RNAi-NS2.1* (on the 2nd chromosome) and mRFP-RpS6 (our F1 line on the 3rd chromosome) was previously described (Chapter 2, Rosby et al., 2009). All fly lines were maintained on standard fly food at room temperature (22-24° C).

Microscopy

For *da-GAL4 > UAS-RNAi-NS2.1; UAS-GFP-RpS6*, tissues were fixed in a formaldehyde-containing buffer as described previously (Cui and DiMario, 2007) before fluorescence microscopy. We used a Zeiss Axioskop equipped with a SPOT SE digital camera and software (Diagnostic Instruments) for conventional fluorescence microscopy. *RNAi-NS2/ey-Gal4* flies were imaged using a Lumar.V12 SteREO (Zeiss) microscope fitted with an Axiocam MRc5 (Zeiss) camera.

Immuno-precipitation and Mass spectroscopy

Transfected embryonic Schneider 2 (S2) cells expressing FLAG tagged NS1 were heat shocked at 37° C for 1 h to expressed FLAG-NS1. These cells were harvested and centrifuged for 5 min. The supernatant was discarded and 50 µl of protease inhibitor (Sigma) was added to the cells. The cells were homogenized and then lysed in 1 ml of lysis buffer (50mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% TRITON X-100). The cell debris was removed by micro-centrifuging the sample at 5000X g for 10 min. The liquid with protein extract was added to the anti-FLAG M2 affinity resin (Sigma), which was washed twice before use by TBS (50 mM Tris HCl, with 150 mM NaCl, pH 7.4). The protein extract with the anti-FLAG M2 affinity gel was incubated at 4° C for 4 h to overnight. After incubation, the sample was centrifuged and the supernatant was discarded. The resin (FLAG-NS1 bound to the anti-FLAG resin) was washed by TBS twice. FLAG-NS1 was eluted by SDS-PAGE 2X sample buffer (125mM Tris HCl, pH 6.8, with 4% SDS, 20% (v/v) glycerol, and 0.004% bromphenol

blue). Finally, the sample was boiled for 5 min and micro-centrifuged for 5 min. The supernatant was resolved by standard SDS-PAGE gel.

Trypsin (Sigma) was used for in-gel digests. Bands of interest were cut carefully by razor blade, and then placed in a siliconized Eppendorf tube, which was prewash with 100 μ l of a 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile solution. Gel pieces were covered by 200 μ l of 200 mM ammonium bicarbonate with 40% acetonitrile and incubated at 37° C for 30 min. The incubation step was repeated twice. The gel pieces were then dried in a Speed Vac for 30 min. Twenty μ l (0.4 μ g of trypsin) of trypsin solution and 50 μ l of 40 mM ammonium bicarbonate in 9% acetonitrile solution were added to the gel pieces and incubated at 37° C overnight. After incubation, the liquid containing the extracted tryptic peptides was transferred to a new tube. The solution was completely dried in a Speed Vac. The MALDI-MS analysis was done by the Chemistry Department, LSU.

Immuno-blots

Standard SDS-PAGE was used for western blots. The proteins were blotted to nitrocellulose for 40 minutes using a semidry system (Bio-Rad Laboratories). Blots were blocked for 1 h in 3% nonfat dry milk that had been reconstituted in 0.9% NaCl (wt/vol), 100 mM Tris, pH 7.4, and 0.1% Tween 20 (TTBS). Blots were probed with the chicken polyclonal antibody described in Chapter 2 diluted to 1/1000 in TTBS. An affinity-purified, peroxidase-conjugated goat anti-chicken IgY (Pierce Chemical) diluted 1/250 with TTBS was used as secondary antibody.

Results

NS2 depletion caused RpS6 accumulation in the nucleoli

Chapter 2 described how *Drosophila* NS2 is required for the release of the 60S subunit from nucleoli. To test whether NS2 is also required for small ribosomal subunit release, we established larvae that expressed RFP-RpS6 either with normal NS2 levels (*da-GAL4 > UAS-GFP-RpS6*) or with NS2 depleted by RNAi expression (*da-GAL4 > UAS-RNAi-NS2.1; UAS-GFP-RpS6*). In the control larvae with normal NS2 levels, GFP-RpS6 mainly accumulated in the cytoplasm of 2nd instar midgut cells (Figure A.1, g), Malpighian tubule cells (Figure A.1, i) and 3rd instar fat body cells (Figure A.1, k), presumably within small ribosomal subunits. Like GFP-RpL11, RFP-RpS6 was barely detectable in the nucleoli of these cells, suggesting a minimum retention of RFP-RpS6 in nucleoli during normal ribosome biogenesis. These control larvae were perfectly healthy, and all the larvae developed into adult flies, indicating that exogenous expression of RFP-RpS6 had no significant harm. In contrast, however, RFP-RpS6 accumulated within nucleoli when NS2 was depleted by RNAi (Figure A.1, a-f). All larvae depleted of NS2 died in the 2nd and 3rd larval instar stages.

Loss of NS2 disrupted eye development

To express NS2-RNAi in the *eyeless* expression pattern, we crossed the *ey-Gal4/CyO* line with the homozygous *UAS-RNAi-NS2.1* line (abbreviated as RNAi-NS2) (Figure A.2). F1 progeny would have two genotypes, *RNAi-NS2/ey-Gal4* and *RNAi-NS2/CyO*. All *RNAi-NS2/ey-Gal4* F1 progeny had straight wings and expressed RNAi-NS2. These flies showed at least one malformed eye. In

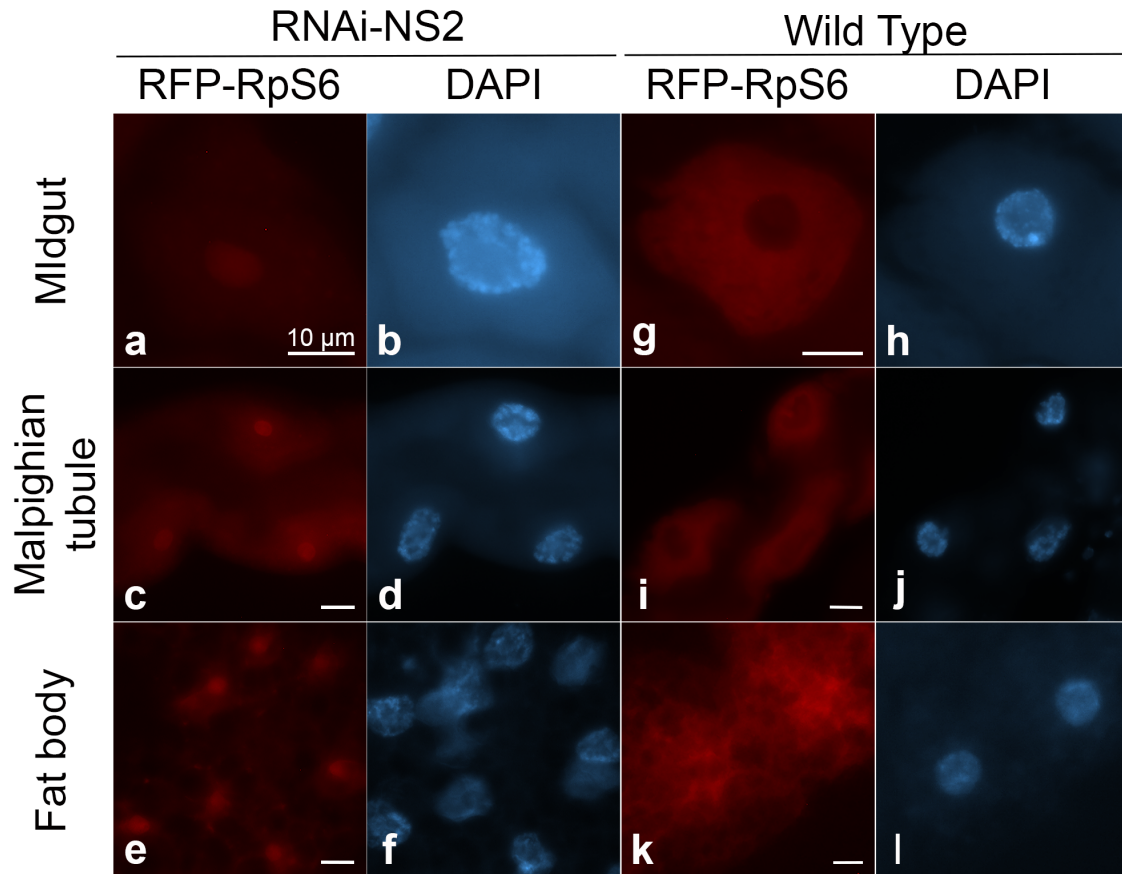


Figure A.1 Loss of NS2 by RNA interference inhibited release of the 40S subunit as marked with RFP-RpS6 from nucleoli. a-f Midgut, Malpighian tubule and fat body cells from second instar transgenic larvae lacking NS2 (*da-GAL4* > *UAS-GFP-RpS6*; *UAS-NS2 RNAi*) accumulated RFP-RpS6 within their nucleoli. g-l Midgut, Malpighian tubule cells from second and fat body cells from third instar transgenic larvae but expressing NS2 (*da-GAL4* > *UAS-RFP-RpS6*; +) showed preferential cytoplasmic labeling due to RFP-RpS incorporated into ribosomes.

contrast, all *RNAi-NS2/ey-Gal4* progeny had curly wings but normal eyes, and we used these siblings as controls.

Mass spectroscopy results of NS1

We expressed FLAG-NS1 in *Drosophila* S2 cells, and immuno-precipitated FLAG-NS1 using anti-FLAG resin. The SDS-PAGE gel stained with

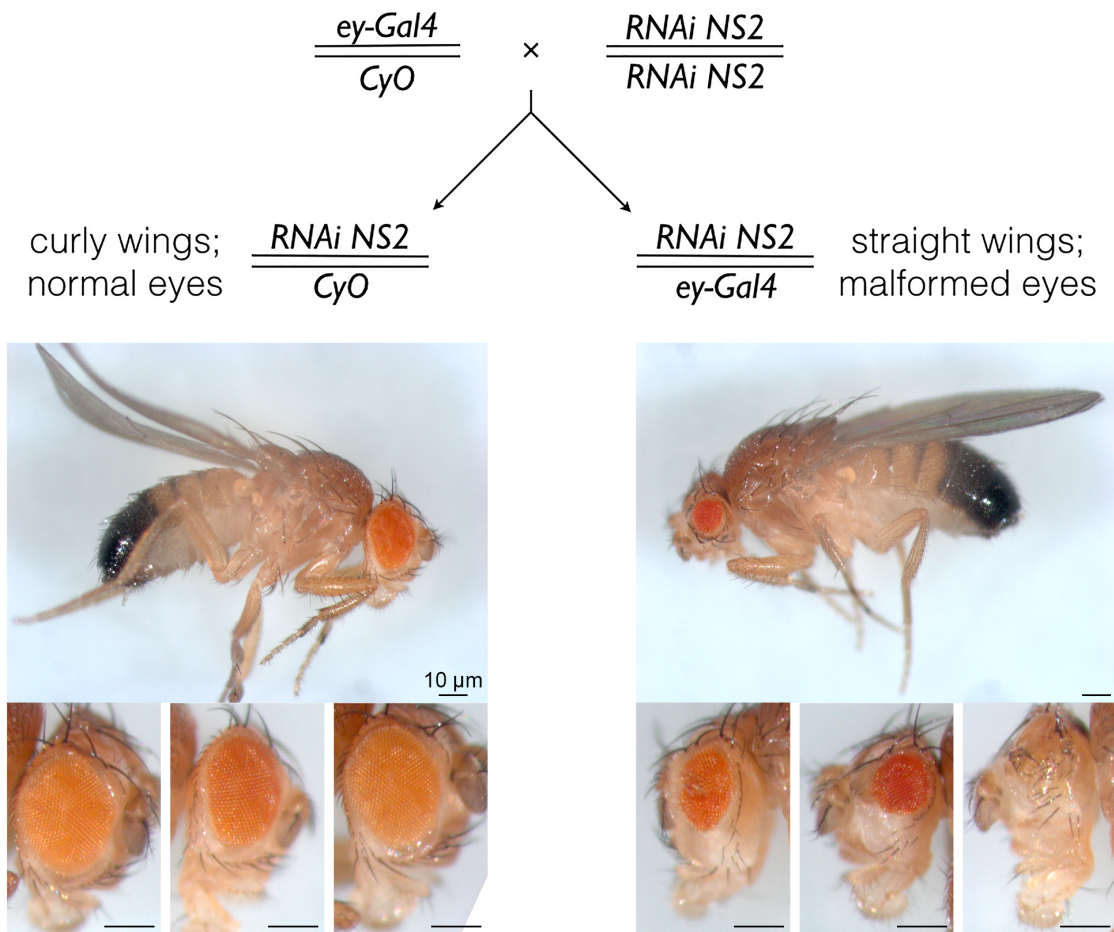
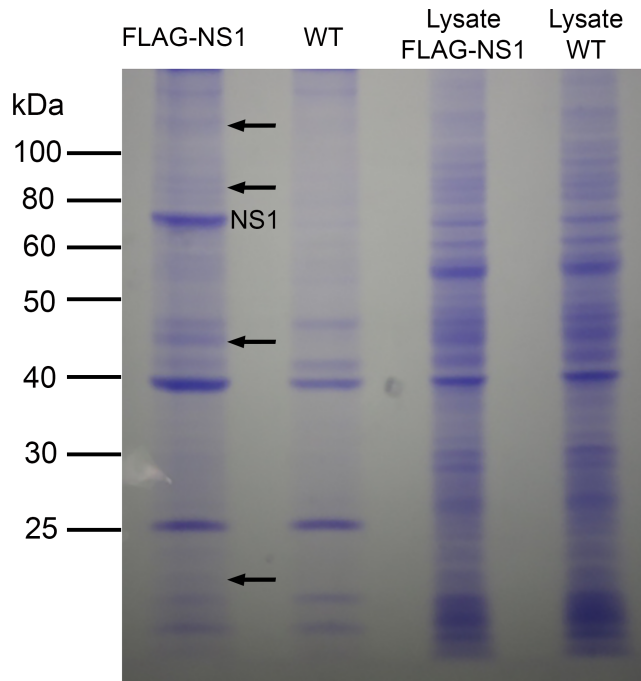


Figure A.2 Expression of RNAi-NS2 in *eyeless* pattern. *ey-Gal4/CyO* flies were crossed to *UAS-RNAi-NS2* homozygous flies at 27-28° C to knockdown NS2. Flies expressing RNAi-NS2 (*RNAi-NS2/ey-Gal4*) were separated from sibling controls (*RNAi-NS2/CyO*) due to the absence of curly wings. Loss of NS2 leads to defect eyes to varying degrees. Bar, 200 µm for all photos.

Coomassie brilliant blue showed that FLAG-NS1 was concentrated after immuno-precipitation (IP) (Figure A.3, a, lane 1) compared to the control (Figure A.3, a, lane 2). Western blots using chicken anti-NS1 confirmed that FLAG-NS1 was purified by IP (Figure A.3, b). There were many proteins co-immunoprecipitated with FLAG-NS1, and some examples are shown by arrows.

a IP anti-FLAG



b Anti-NS1

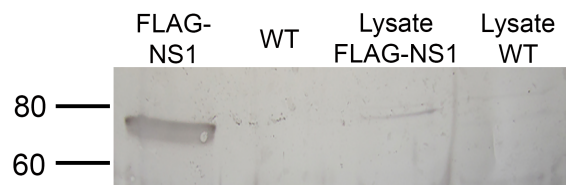


Figure A.3 a A Coomassie stained SDS gel showing NS1 was purified and concentrated from transfected S2 cells expressing FLAG-NS1 after immuno-precipitation. b Companion immunoblots were probed with anti-NS1. The band (between 60 and 80 kDa) in the first lane confirms the NS1 band in Coomassie stained gel.

To identify the co-precipitated proteins that may be the candidate binding partners of NS1, we cut out the bands carefully and prepared them for mass spectroscopy. The results from mass spectroscopy showed that NS1 did not only interact with ribosomal large subunit proteins, it also interacted with ribosomal small subunit

proteins (Table A.1). More importantly, Exportin 1 (Crm1), a nuclear export factor, was in the list of candidate binding proteins.

Table A.1 Possible binding partners of *Drosophila* NS1

Name of Proteins	Function
40S ribosomal protein S3	Ribosome constituent
40S ribosomal protein S4	Ribosome constituent
40S ribosomal protein S6	Ribosome constituent
40S ribosomal protein S7	Ribosome constituent
40S ribosomal protein S9	Ribosome constituent
40S ribosomal protein S14a	Ribosome constituent
40S ribosomal protein S16	Ribosome constituent
40S ribosomal protein S17	Ribosome constituent
40S ribosomal protein S20	Ribosome constituent
40S ribosomal protein S27a	Ribosome constituent
60S ribosomal protein L7a	Ribosome constituent
60S ribosomal protein L8	Ribosome constituent
60S ribosomal protein L13a	Ribosome constituent
60S ribosomal protein L17	Ribosome constituent
60S ribosomal protein L18	Ribosome constituent
60S ribosomal protein L31	Ribosome constituent
60S ribosomal protein L36	Ribosome constituent
Exportin-1	Ribosome export
Putative homeodomain transcription factor	?
Nuclear cap-binding protein subunit 1	?
Protein dead ringer	?
Lysine-specific demethylase NO66	?
rRNA 2'-O-methyltransferase fibrillarin	?
ATP-dependent RNA helicase WM6	?
DEAD box protein/ATP-dependent RNA helicase Protein	?
phosphatase PP2A 55 kDa regulatory subunit	?

Conclusion

Here we show that NS2 may be involved in ribosomal small subunits (SSU) maturation/release, and that NS2 is required for multiple tissue

development. In addition, NS1 may interact directly with CRM1. These are all preliminary experiments that need to be verified in the future.

Appendix B

Chromosome Maps of Transgenic Stocks

Insert	Line	Chromosome	Phenotype
RNAi-NS2	1	2	Normal
RNAi-NS2	2	2	
RNAi-NS2	3	3	
RNAi-NS2	4	2	Normal
RNAi-NS2	5	Not 3	Homozygous lethal
RNAi-NS2	6	3	Homozygous lethal
RNAi-NS2	7	3	Normal
RNAi-NS2	8	?	Homozygous lethal
RNAi-NS2	9	Not 2	Normal
RNAi-NS2	10	2	Homozygous lethal
RNAi-NS2	11	?	Normal
RNAi-NS2	12	2	Normal
RNAi-NS2	13	2	Normal
RNAi-NS2	14	2	Normal
RNAi-NS2	15	2	Normal
RNAi-NS2	16	Not 2	
RNAi-NS2	17	2	Normal
RNAi-NS2	18	Not 2	Homozygous lethal
GFP-NS2	1	X	Normal
GFP-NS2	2	3	Normal
GFP-NS2	3	2	Homozygous lethal
GFP-NS2	4	2	
GFP-NS2	5	2	
GFP-NS2	6	2	Homozygous lethal

Vita

Yubo Wang is the only child of Jimin Sun and Qimin Wang. He graduated with a Bachelor of Sciences degree from Ludong University in Yantai, China, in July of 2006. He graduated with a Master of Sciences degree from Shanghai Ocean University in Shanghai, China in July 2009. He started his doctoral work in Louisiana State University in Baton Rouge, Louisiana in the fall of 2009. Yubo is a candidate to graduate with the degree of Doctor of Philosophy in biological sciences in December of 2015.